SIGNAL TRANSDUCTION

THIRD EDITION

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Biography

IJsbrand Kramer is a professor at the University of Bordeaux, working in the European Institute of Chemistry and Biology (IECB). He holds a bachelors and masters degree in biomedicine from the University of Utrecht, the Netherlands, with a one year research-excursion in the Department of Cell Biology at the University of Liverpool, the UK. He did his PhD at the University of Amsterdam, in the Central Laboratory of Blood Transfusion Services (Stichting Sanquin) and worked as a postdoctoral fellow at the Hubrecht Laboratory in Utrecht and at the University of Washington in Seattle. He then took a lecturer position at the Department of Pharmacology at University College London, where he taught signal transduction (with Bastien Gomperts and Peter Tatham) and pharmacology. Both teaching activities have been documented in textbooks: Signal Transduction (three editions) and Receptor Pharmacology (CRC Press/Taylor Francis Group, three editions). Most of his research centers on the theme of inflammation, starting with neutrophils and the NADPH oxidase, synovial fibroblasts and destruction of the joint and more recently podosomes formation and extracellular matrix destruction in vascular endothelium. He moved to the University of Bordeaux for family reasons and switched from pharmacology to cell biology, with a strong contribution to an introductory course for first-year university students. Given the important teaching load and the general low level of student engagement in higher education, he started to investigate the reasons for student failure (finding out about their expectations and attitudes) and the role of images and animations in comprehension. Scientific publications, Web-based multimedia resources, and dramatically enhanced retention rates (from 33% to 85%) are the fruits of these activities. At the same time, he organized with University College London and Universitat Pompeu Fabra, Barcelona, summer schools on Receptor and Signaling Mechanism. He has been codirector of two European Programmes (Interbio and Transbio) that aimed at enhancing industrial innovation in the biomedical sector in the South West European region (SUDOE). More information about his teaching and pedagogical-research activities can be found at www.cellbiol.net.

Preface

The third edition differs considerably from the earlier editions in that the book is no longer separated in two parts, the first providing the nuts and bolts of what might be termed classical signal transduction. In fact, this classification does not really apply for two reasons. First, for students everything is as new as the latest scientific article is for a teacher and dividing between classical or nonclassical is not really making the subject clearer and might even pretend that cells employ classical (important) and not-so classical (less important) mechanisms that bring about changes in their metabolism, gene expression, secretion rate, contraction state, and so on. Second, with the recent structural revelations of G protein-coupled receptor and the action of biased agonists, classical signal transduction has suddenly lost its classical touch and has become very modern. Instead, an introductory chapter has been added in which a number of principles are outlined common to many signal transduction events, and these are placed in the context of the most "Nobel", the most classic, of all pathways: adrenaline to glycogen phosphorylase. While the previous editions were written by three authors and rewritten by Bastien Gomperts, so that it appeared as written by one mind, one hand, this edition also differs in that it has been written by one hand only (or, less poetically, typed by two hands on a keyboard). The two "greybeards" have pulled out after publication of the second edition and, very sadly, Bastien Gomperts has passed away in October 2013. If, from the previous editions, you appreciate the writing style, the wit, and the anecdotes, bearing from some unusual sources, much of the credit goes to him. He was an inspiring mentor indeed.

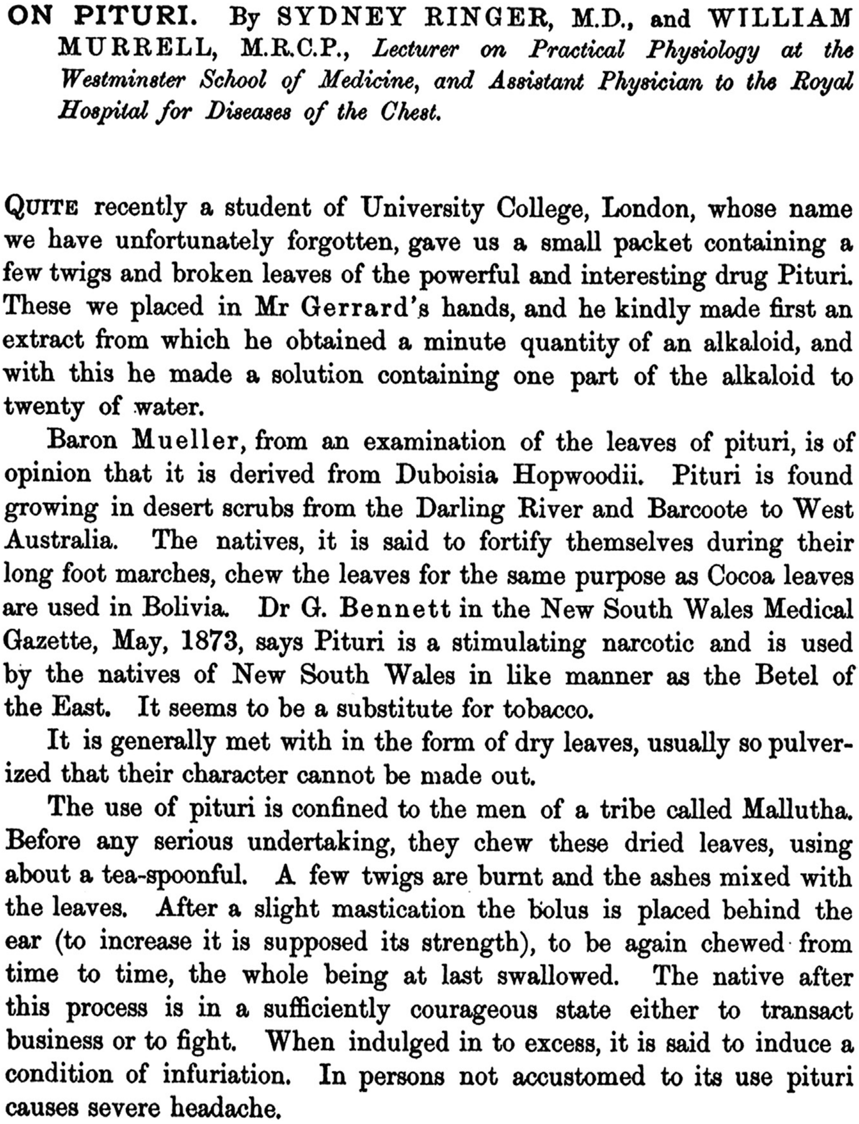
With a few exceptions, for instance the chapter on protein phosphatases, each chapter now has a theme of its own, around which specific aspects of signal transduction pathways are developed. For teaching purposes, Table 1-1 attempts to give a short overview of each chapter's subjects and highlights, so that, depending on the pathway to be explored, a relevant (suitable) context can be selected. Although there still is a gradual build-up of the subject, where later chapters make reference to earlier chapters, each chapter could stand on its own. Naturally, the signaling aspects highlighted are not necessarily unique to the context in which they are developed, but it allows teachers (and students) to tell a story rather than just listing a sequence of signaling events (Kramer and Thomas, 2006). As a consequence, this edition contains more on cell biology, physiology, pathology, and immunology. By providing precise examples, embedded in precise contexts, the book offers the possibility to integrate signaling knowledge into the above-mentioned disciplines and, therefore, facilitates a constructive approach to teaching (for more information, see http://www.cellbiol.net/docs/Constructive_teachingKramer.pdf).

Again, there has been no attempt to be comprehensive and certain important topics that well qualify for inclusion in this book, such as signals initiated by damaged DNA or unfolded proteins, are conspicuous only by their absence. Although the book touches the leading edges of the subject, it also endeavors to provide an elementary basis with some historical background to all the topics covered. The "prologue" has been extended considerably with new information about the first observations of "irritability," a phenomenon that qualifies as "an essential element of the living" (besides template replication and metabolism). Historical background not only provides a broader insight into the subject and pays tribute to the wisdom of our forebears, whose freedom of thought and sometimes serendipitous discoveries in the nineteenth and early twentieth centuries led to the creation of the modern sciences, for certain people it may also provide meaning to the numerous odd names, abbreviations, and acronyms (collectively named symbols) that riddle the book. For unexplained symbols the reader is encouraged to consult UniProt (paragraphs "function" and "names & taxonomy") or relevant Wikipedia articles. Besides the chapter about intracellular calcium, the book does not reveal a good deal of experimental techniques. A lack of time and a growing complexity of technology are to blame. Moreover, experience tells that explaining technology is revealing for learners who already master the cellular context and signaling events but tend to mystify matters, because of a substantial increase in cognitive burden, when learners are still struggling with the molecular mechanisms that drive the pathways.

In preparing the book, I have had the benefit of advice and opinions from many friends and colleagues. These include (in order of their first name) Alan Hall (New York), Alasdair Gibb (London), Bob Weinberg (Cambridge, USA), Bob Lefkowitz (Durham, USA), Bruno Klaholz (Illkirch), Carsten Hoege (Dresden), Chris de Graaf (Amsterdam), Christopher Glass (San Diego), David Armstrong (Durham, USA), David Strutt (Sheffield), Filip van Petegem (Vancouver), Geerten Vuister (Leicester), George Mosialos (Thessaloniki), Graham Dunn (London), Jean Dessolin (Bordeaux), Jeff Saucerman (Charlottesville), Jennifer Lippincott-Schwartz (Bethesda), Jürgen Knoblich (Vienna), Karin Rittinger (London), Karl Matter (London), Maria Schumacher (Durham, USA), Marian Joëls (Utrecht), Marina Gloukova (Paris), Mark Dell'Acqua (Aurora), Matthew Gold (London), Michel Laguerre (Bordeaux), Miho Lijima (Baltimore), Mingjie Zhang (Hong Kong), Peter van Haastert (Groningen), Purna Joshi (Toronto), Roel Sterckx (Cambridge, UK), Romuald Nargeot (Bordeaux), Sander van den Heuvel (Utrecht), Shiva Malek (South San Francisco), Stuart Firestein (New York), Yohanns Bellaiche (Paris), Vadim Asharvsky (Durham, USA), Wai Leong (Singapore), and Wei-Min Shen Los Angeles. Special gratitude also goes to all authors, curators, Website developers, and

technicians who contributed and continue to contribute to an increasing number of outstanding annotated databases (UniProt, PubMed, OMIM, UniGene, GenomeNet, HGNC, PhosphoSite, and so on) and Wikis. These databases have become key sources of information for research as well as education. I encourage students to go out on the web!

In acknowledgment of their contribution I offer the following quotation by one of the pioneers of signal transduction (Figure 1-1; Ringer and Murrell, 1878).



ON PITURI. By **SYDNEY RINGER**, M.D., and **WILLIAM MURRELL**, M.R.C.P., *Lecturer on Practical Physiology at the Westminster School of Medicine, and Assistant Physician to the Royal Hospital for Diseases of the Chest.*

QUITE recently a student of University College, London, whose name we have unfortunately forgotten, gave us a small packet containing a few twigs and broken leaves of the powerful and interesting drug Pituri. These we placed in Mr Gerrard's hands, and he kindly made first an extract from which he obtained a minute quantity of an alkaloid, and with this he made a solution containing one part of the alkaloid to twenty of water.

Baron Mueller, from an examination of the leaves of pituri, is of opinion that it is derived from Duboisia Hopwoodii. Pituri is found growing in desert scrubs from the Darling River and Barcoote to West Australia. The natives, it is said to fortify themselves during their long foot marches, chew the leaves for the same purpose as Cocoa leaves are used in Bolivia. Dr G. Bennett in the New South Wales Medical Gazette, May, 1873, says Pituri is a stimulating narcotic and is used by the natives of New South Wales in like manner as the Betel of the East. It seems to be a substitute for tobacco.

It is generally met with in the form of dry leaves, usually so pulverized that their character cannot be made out.

The use of pituri is confined to the men of a tribe called Mallutha. Before any serious undertaking, they chew these dried leaves, using about a tea-spoonful. A few twigs are burnt and the ashes mixed with the leaves. After a slight mastication the bolus is placed behind the ear (to increase it is supposed its strength), to be again chewed from time to time, the whole being at last swallowed. The native after this process is in a sufficiently courageous state either to transact business or to fight. When indulged in to excess, it is said to induce a condition of infuriation. In persons not accustomed to its use pituri causes severe headache.

FIGURE 1-1

Of course, the authors of this paper would themselves never have recognized the expression signal transduction, and it would be a further 100 years before it made its appearance in the biological literature. The sensations brought about by pituri, an alkaloid that Ringer and Murrell described as sharing some of the pharmacological properties of atropine (courage, infuriation, frustration, and headaches), are not dissimilar to those experienced in the writing of this book. Indeed, they will be familiar to many students and investigators in this and other fields of research. However, we should not take this too far. When Ringer (1879) tested the effects of the application of pituri on four men, he noted that it also causes drowsiness, faintness, pallor, giddiness, hurried and superficial breathing, dilates the pupil, produces general weakness with convulsive twitchings, and antagonizes the action of muscarin on the heart. Unlike atropine, it produces sickness and increases the salivary secretion in large doses copiously, the breathing becomes quick and shallow, and general weakness ensues. Reading all this, it leads one to wonder who, among their students, colleagues, and servants, may have offered themselves up as willing, or less than willing, guinea pigs in the furtherance of scientific research. Ringer and his friends apparently preferred to eschew membership of the very honorable brotherhood of self-experimenters, of which the more famous members include Sir, Humphry Davey, who breathed nitrous oxide as well as other more noxious gases, John Scott Haldane, who too inhaled lethal gases; and more recently Barry Marshall, who has swallowed a culture of *Helicobacter pylori* to show that it caused stomach ulcers and who with Robin Warren was awarded the Nobel Prize in Physiology or Medicine in 2005. Another member of this fraternity, Charles Eduard Brown-Sequard, figures prominently in “prologue” chapter.

NOTES

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See the companion website: http://booksite.elsevier.com/9780123948038

For protein structural data we have made use of

The Protein Data Bank: Berman et al. (2000).

Protein structures have been generated using PyMol (education version), a molecular visualization system on open source foundation, maintained and distributed by Schrodinger.

References

We have tried to provide original text sources to nearly all the statements, experiments, and discoveries discussed. The main reason for this is that we ourselves have necessarily had to extend the treatment of nearly all the topics presented far beyond the areas of our own experience or expertise. Thus, comprehensive lists are there to provide us with some sort of reassurance that what we have written has not simply been conjured out of the air. Also, because we have made a particular feature of presenting original historical source material by quotation, which necessarily required referencing, it seemed logical also to include literature references to modern sources as well. Thus we hope that this book may serve as a valuable resource, in the manner of a basic literature review, for anyone wanting to explore further.

Protein symbols (gene products)

We have named proteins according to the symbols agreed upon in the HUGO gene nomenclature database (HGNC) (www.genenames.org). Similar symbols have been adopted by protein databases such as UniProt. Some of the new symbols are simply awkward, so different from conventional names that they are even not recognized by scientists who made major contributions to the field, and when they really are uncommon alternative more conventional names are provided, but from a pedagogical point of view it is vital that students can search the web with unique (unambiguous) symbols and find out about the relevant proteins (and genes) themselves. And remember, certain symbols may be more familiar to experts in the field, for students they are all the same: new and often gruesome.

References

Kramer, I.M., Thomas, G., Spring 2006. Meeting report: teaching signal transduction. CBE Life Sci. Educ. 5, 19–26.

Ringer, S., Murrell, W., 1878. On pituri. J. Physiol. 377–383.

Ringer, S., 1879. On the action of pituri on man. Lancet 290–291.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The protein data bank. Nucleic Acids Res. 28, 235–242. http://www.rcsb.org/pdb/.

TABLE 1-1 Contexts, pathways, subjects, and proteins/molecules elaborated in different chapters

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
1. Prologue: Signal transduction from an historical perspective	An account of how the term "signal transduction" entered biomedical research and how stimulus–response coupling, hormones, neurotransmitters, growth factors, and their receptors were brought to light	• Alfred Gilman • Martin Rodbell • Thomas Henry Huxley • Steve Grand • Charles Edouard Brown-Séquard • Henry Hallett Dale • Otto Loewi • George Oliver • Edward Scharpey-Schäfer • Ernest Henry Starling • Willam Maddock Baylis • CONRO, a self-reconfigurable robot • Paul Ehrlich • John Newport Langley • Francis Peyton Rous • Rita Levi-Montalcini • Stanley Cohen • Alexis Carrel • Howard Temin • Renato Dulbecco
2. An introduction to signal transduction Context: Adrenalin-mediated activation of glycogen phosphorylase in striated muscle	• Signaling pathways serve to create symbolic representations of the cellular environment • First messengers, description of hormones, growth and differentiation factors, cytokines, inflammatory mediators, vasoactive agents, neurotransmitters	• Aph(2′)-Ib, aminoglycoside-2 phosphotransferase, *Escherichia coli* (ancient) protein kinase fold, structural composition, conserved residues, αC helix • Docking site, substrate-binding motifs in MAPKinase, D motif, and DEF motif, examples of substrates, sequence of the transcription factor ELK1

	• Signaling in context, the signal itself is ambiguous but context, cell type, and cell condition, determine the outcome • Receptor–ligand concept, classic pharmacology, different receptor types • Signaling mechanisms, the sequence of receptor–transducer–effector–second messenger • Adrenaline to glycogen phosphorylase, pioneer studies in signal transduction, detailed transcription of the pathway as operated in muscle • Nobel laureates associated with the adrenaline to glycogen phosphorylase pathway • Wired allostery, signal integration, and thoughtful decisions • Posttranslational modifications, a broad overview • Feedback mechanisms, modeling of EGFR-mediated activation of MAP-kinase	• Edmond Fischer and Edwin Krebs, Nobel Laureates 1992 (protein phosphorylation, glycogen phosphorylase) • Eicosanoids, molecular composition, prostaglandin, prostacyclin, thromboxane, leukotriene, anandamide • GNAI1, Gαi subunit of heterotrimeric G-protein, structural composition, conserved residues, GTP-binding pocket, GαRAS and alpha-helical segment • H3F3A, histone-3.3, posttranslational modifications, acetylation, crotonylation, methylation, and phosphorylation • HRAS, monomeric G-protein, molecular structure, position of conserved residues, mechanism of nucleotide exchange and GTP hydrolysis, GTPase cycle, switch regions, farnesylation • Cellular communication, modes of, endocrine, paracrine, juxtacrine, synaptic, and autocrine • Pharmacology, agonist, antagonist, inverse agonist, receptor–ligand interaction, affinity, dose–response curve, receptor number, and signal sensitivity • Phosphoenolpyruvate-dependent phosphotransferase system in bacteria • Phosphorylation, consequences for activity and subcellular localization of proteins, potential phosphate donors, ATP favorite but not exclusive

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
		• Phospho-amino acids, most occurring phosphate acceptors in proteins, stability of phospho-ester, phospho-ramidate, and phospho-anhydride bond • Phosphoryl transferase, phosphorylation, protein kinase, catalytic mechanism, role of conserved residues • PRKACA, protein kinase A, catalytic subunit, serine/threonine kinase, structural composition, conserved residues, N-lobe, C-lobe, signature sequences, αC-helix, catalytic mechanism • Protein kinases, classification, serine/threonine, tyrosine and dual-specificity protein kinases, their mode of regulation • Protein phosphatases, classification, serine/threonine, tyrosine and dual-specificity phosphatases, structural composition, catalytic mechanisms • PYGM, glycogen phosphorylase muscle form, structural composition, dimer, hydrolysis of glycogen and phosphorylation of glucose (glucose-1-phosphate), allosteric regulation of enzyme, phosphorylation by glycogen phosphorylase kinase on serine-14, change in configuration, tense to relaxed state • RASA1, RAS GTPase-activating protein, RAS–GAP domain, molecular structure, catalyzes hydrolysis of GTP • Receptors, classification, ligands, overview of different types • SOS1, RAS-nucleotide exchange factor, GEF, Cdc25 domain, molecular structure, αH helix, mechanism of removal of GDP from nucleotide pocket • Two-component signaling and environmental sensors in bacteria

3. Regulation of muscle contraction by adrenoceptors Contexts: a. Cardiac muscle contraction b. Smooth muscle contraction Pathways: a. Action potential, voltage sensitive Ca^{2+} channels, Ca^{2+}-induced-Ca^{2+} release (ryanodine receptor), intracellular free Ca^{2+} release, troponin, myosin–actin cross-bridge cycle b. Adrenoceptor β1, heterotrimeric G-proteins (Gαs), adenylyl cyclase, cAMP, and protein kinase A c. Heterotrimeric G-proteins (Gαs), beta-adrenergic receptor kinase, arrestin, switch to MAP-kinase pathway, non-receptor tyrosine kinases, and protein kinase B (AKT)	• Catecholamines (adrenaline, noradrenaline, dopamine), molecular composition • Central and autonomic–peripheral nervous system, anatomy, and neurotransmitters involved • Adrenoceptors, adrenergic receptors, history, classification • Agonist, inverse agonist, biased agonist, and antagonist, mode of action, examples • Muscle contraction, cardiac and smooth muscle, anatomy, myosin–actin cross-bridge cycle • G-protein effectors, adenylyl cyclase and phospholipase C, second messengers cAMP, and diacyglycerol/IP$_3$ • Noradrenaline-mediated control of cardiac-muscle contraction: ADRB1 (β1AR), GNAS (Gαs), ACDY5, cAMP, PRKACA (PKA), CACNA1C (voltage-sensitive Ca^{2+} channel), RYR2, TNNC1, TTNI3 (troponin), myosin–actin cross-bridge cycle, PLN (phospholamban), ATP2A2 (SERCA2, Ca^{2+} pump), KCNQ1 (K^{+} channel), ATP2B2 (Ca^{2+} pump) • G protein-receptor kinases (GRK), among which ADRBK1 and GRK6 • Arrestins and arrestin-dependent signaling (among others, SRC, RAF–MAPK, AKT1, NFKB) • Feedback mechanisms, modeling of noradrenaline-mediated molecular events	• ADCY, adenylyl cyclase, structure, catalytic mechanism, production of cAMP, family members, mode of activation, and inhibition • ADRA, ADRB, adrenoceptor, adrenergic receptor, ligand-binding site, conformational changes, energy landscape, coupling to heterotrimeric G-proteins or G protein-receptor kinases (GRK) • adrenalin, composition, its agonists, inverse agonists, biased agonist, and neutral antagonists • ARRB, arrestin proteins, structure, family members • GRK6, G protein-receptor kinase, structure, function, family members, mechanism of attachment to GPCR • Heterotrimeric G-proteins, mechanism of nucleotide exchange, guanine-exchange function (GEF) of seven-membrane-spanning receptors (GPCR) • ITPR1, IP$_3$ receptor, cryo-electromicroscopy-determined structure, molecular detail of the IP$_3$-binding site • PLC, phospholipase C, domain architecture, structural composition, TIM barrel, X/Y-linker, catalytic mechanism, production of diacylglycerol and inositol-1,4,5-trisphosphate (IP3), family members, family tree, control of PLCB3 by GNAQ (Gαq)

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
4. Cholinergic signaling and muscle contraction Contexts: a. Skeletal muscle contraction b. Cardiac muscle contraction and relaxation c. Smooth muscle contraction and relaxation Pathways: a. Nicotinic acetylcholine receptor, voltage sensitive Na^+-channel, membrane depolarization, voltage sensitive Ca^{2+}-channel, Ca^{2+}-induced-Ca^{2+}-release (ryanodine receptor), intracellular free Ca^{2+}, troponin, myosin-actin cross-bridge cycle b. Muscarinic acetylcholine receptor-3, G protein-coupled receptors, heterotrimeric G-proteins (Gαi, Gαq, Gα12, and Gβγ), - Pacemaker, cardiomyocyte, Gαi, adenylyl cyclase down, Gβγ-gated K^+-channel conductivity, cAMP-gated Na^+ channel	• Acetylcholine, history, composition, synthesis, and breakdown • Synapse, active zone, vesicle docking, membrane fusion machinery • Muscarinic and nicotinic receptors • Nicotinic receptor (type IV), neuromuscular junction, membrane depolarization and skeletal muscle contraction • Muscarinic receptor (M2) and vagal (parasympathetic) control of cardiac force and rhythmicity • Cyclic nucleotide phosphodiesterase (PDE) and pathway control • Muscarinic receptor (M3), bronchial smooth muscle contraction, mucus production, salbutamol, and asthma • Nitric oxide, history, NO synthase, smooth muscle relaxation • Guanylyl cyclases, cGMP, protein kinase G (PRKG) • PDE5A, cGMP-specific 3′,5′-cyclic phosphodiesterase-5A, in corpus cavernosum, inhibition by sildenafil (Viagra) and penile erection • Examples of ionotropic and metabotropic receptors for neurotransmitters	• ACh, acetylcholine, composition, synthesis, breakdown, agonists, and antagonists • CHRN, nicotinic acetylcholine receptors, channel topology, structural composition, subunits, control of pore permeability (gating), detail ligand binding pocket, aromatic cage, comparison with AChBP, snail protein • CHRM, muscarinic acetylcholine receptors, structural composition, bitopic ligand-binding site • GUCY, guanylyl cyclase (soluble), family tree of guanylyl cyclases, structural composition, α- and β-subunit, catalytic mechanism • Inward rectifier K^+ channel (Kcnj6) in complex with Gβγ • NOS (nitric oxide synthase), structural composition, prosthetic groups, catalytic mechanism • PDE, cyclic nucleotide phosphodiesterase, structure, cyclic-nucleotide binding (cAMP versus cGMP), regulation of activity • PDE5A, cGMP-specific 3′,5′-cyclic phosphodiesterase-5A, target of sildenafil (Viagra), molecular structure with inhibitor • PRKG1, protein kinase G, molecular structure, regulation by cGMP

- bronchial Smooth muscle, Gαq, phospholipase Cβ, phosphatidylinositol-4,5-bisphosphate (PIP2), inositol-1,4,5-trisphosphate (IP$_3$), diacylglycerol, IP$_3$ receptor, intracellular-free Ca^{2+}, smooth muscle myosin light chain kinase, actin–myosin cross-bridge cycle, protein kinase C, guanine nucleotide exchange factor (ARHGEF1), monomeric GTPase (RHOA), ROCK1, inhibitory subunit of serine/threonine phosphatases c. Muscarinic acetylcholine receptor-3 endothelium, G protein-coupled receptors, phospholipase Cβ, phosphatidyl-4,5-bisphosphate, diacylglycerol, inositol-1,4,5-trisphosphate, IP$_3$-receptor, intracellular free Ca^{2+}, NO synthase, nitric oxide d. Nitric oxide, smooth muscle cell, soluble guanylyl cyclase, cGMP, protein kinase G, effect on regulatory subunit of serine/threonine phosphatase and dephosphorylation of smooth-muscle myosin-regulatory light chain, myosin–actin cross-bridge cycle		

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
5. Sensory signal processing: visual transduction and olfaction Contexts: a. Vision and retinal photoreceptors b. smell and olfactory epithelium Pathways: a. G-protein coupled receptors (rhodopsin), heterotrimeric G-protein (Gαt or transducin), guanylyl cyclase, removal cyclic cGMP, nucleotide-gated Na^+/Ca^{2+} ion channel, voltage-gated Ca^{2+} channel, neurotransmitter release (glutamate), G protein-coupled kinase, arrestin, transducin GAP-complex b. G protein-coupled odorant receptors, Gαolf, adenylyl cyclase, production cAMP, cAMP-sensitive Na^+/Ca^{2+} channel, Ca^{2+}/calmodulin-gated Cl^- channel, membrane depolarization	• Eye, development, anatomy, retina, cell types • Rods and cones, disks, rhodopsin, absorption spectra, color vision, 11-cis-retinal, all-trans-retinal, vitamin A • Retinal metabolism, retinal-pigment epithelium • Rhodopsin (RHO), metarhodopsin, structure, conformational change of helical bundle, binding of Gαt (transducin) or GRK1 • Effectors, inhibition of guanylyl cyclase GUCY2D, activation phosphodiesterase PDE6, cGMP-sensitive cation channel (CNGA1) • Signal attenuation, GRK1, arrestin (SAG), and transducin GAP complex (PDE6G, GBB5, RGS9) • Light, darkness, adaptation • *Drosophila* compound eye, norpA (PLC), InaC (PRKC), InaD (scaffold) and Trp-channels • Chemosensory organs mouse, fly and human, olfactory bulb, olfactory epithelium • Odorant receptors, GPCR, Gαolf (GNAL), adenylyl cyclase (ACDY3), cyclic nucleotide-gated channel (CNG), Ca^{2+}/calmodulin-gated chloride channel (ANO2) • Excursion on G protein-coupled receptors	• G protein-coupled receptor, GPCR, 7TM-receptor, excursion: classification, topology of transmembrane helical bundle, Ballesteros–Weinstein generic numbering of highly conserved residues, contact network of ligand and of Gα, ligand-binding pockets across the category A (rhodopsin-type) receptors, structure comparison between category A and B (secretin type) • Metarhodopsin II, molecular structure, interaction with effectors, Gαt (transducin) or GRK1 • Retinal, chromophore, 11-cis-retinal lysine, all-trans-retinal, photoisomerization, vitamin A, metabolic pathway, retinal-pigment epithelium • Rhodopsin, opsin +11-cis-retinal, GPCR, different members (OPN1SW, OPN1MW, OPN1LW, RHO), molecular structure of RHO, residues that determine differences in absorption spectra • Transducin GAP complex, molecular structure of GNAT1, PDE6G, RGS9, domain architecture, membrane recruitment via RGS9BP, acceleration of GTP hydrolysis of transducin

6. Intracellular calcium Contexts: a. Muscle contraction b. Neurotransmitter release c. Cell migration Pathway: a. Phospholipase C, inositol-1,4,5-trisphosphate, diacylglycerol, IP$_3$-receptor, Ca^{2+}-induced Ca^{2+}-release (ryanodine receptor), intracellular free Ca^{2+} - Ca^{2+}/calmodulin sensitive smooth muscle myosin light chain kinase, myosin–actin cross-bridge cycle smooth muscle - Synaptotagmin, membrane fusion, voltage-gated Ca^{2+} channel neurotransmitter release b. Protein kinase C, phosphorylation of Rho-dissociation inhibitor - Guanine nucleotide exchange (ARHGEF1), monomeric GTPase (RHOA), Rho-kinase, phosphatase inhibitor (MYPT), smooth muscle myosin activation, myosin–actin cross-bridge cycle - Guanine Nucleotide exchange (TIAM1), monomeric GTPase (RAC1), activation of actin nucleation (WASP/ARP), membrane protrusion	• Calcium-storing organelles and Ca^{2+} transporter • Ca^{2+}-coordination geometry, Ca^{2+}-binding proteins and Ca^{2+} chelators (EDTA, EGTA) • Ca^{2+}-binding domains (EF-hand, C2-domain, P- and C-domain, Calx-β-motif, gelsolin-repeat) • Calmodulin-binding proteins as effectors • Ca^{2+} indicators (from aquorin to genetically encoded indicators) • Ca^{2+}-permeable channels (ITPR, RYR, TPCN, KNCA, TRP, THEM16A, CACNA1, ORAI1) • Store replenishment through ORAI1 (calcium release-operated calcium channel), the Ca^{2+}-sensor STIM1 and the Ca^{2+}-ATPase (ATP2A1) • Ca^{2+} blips, puffs, spikes and waves • Vesicle fusion with membrane, SNARE complex, synaptotagmin, voltage-gated Ca^{2+} channel (CACNA1C) • Cell migration, protrusion, retraction, actin cytoskeleton (arcs and stress fibers) • Chemokine receptor-mediated localized Ca^{2+} oscillations, activation of smooth-muscle myosin light-chain kinase (MYLK) and activation of WAVE/SCAR/WASP-mediated actin-filament nucleation • Michel Abercrombie, a pioneer in cell migration	• Ca^{2+}-binding domains or motifs, molecular structure of EF-hand, C2-domain, Calx-β motif and gelsolin repeat • Ca^{2+}-permeable channels, members, membrane topology • CALM, calmodulin, structural composition, change in conformation upon binding of four Ca^{2+} ions, interaction with Camk2a, Mylk2, and Kcnn2 • CAMK, Ca^{2+}/calmodulin protein kinase, molecular structure of hub, linker and kinase domain, schematic representation of the assembly into a multiprotein complex, control of kinase activity by Ca^{2+} oscillations • GCaMP3, genetically encoded Ca^{2+} indicator, EGFP bound to Ca^{2+}/calmodulin, molecular structure • ITPR, IP$_3$-receptor, cryo-microscopy-derived structure, control of Ca^{2+} conductivity, molecular structure of IP$_3$-binding site • Metal coordination geometry, Ca^{2+}-binding proteins and metal chelators (EDTA, EGTA) • Michel Abercrombie, a pioneer in cell migration • RYR, ryanodine receptor, cryo-microscopy-derived structure, molecular composition of cytoplasmic vestibule, and control of Ca^{2+} conductivity

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Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
7. Bringing the signal into the nucleus: Regulation of gene expression Context: a. Gluconeogenesis Pathway: a. G protein-coupled receptors, heterotrimeric G-protein, Gαs, adenylyl cyclase, cAMP, protein kinase A, cyclic AMP-response element-binding protein (CREB), induction of the gluconeogenic program	• Estimated number of human genes and the central dogma of molecular biology • Starvation and the processes that control gluconeogenesis, role of glucagon and glucocorticoid • Signaling by the glucagon receptor, adenylyl cyclase, cAMP, and regulation of activity of protein kinase A • AKAP, anchoring of protein kinase A to subcellular compartments and scaffolding of signaling complexes, example of AMPA receptor, DLG1, and AKAP79 • CREB, transcription factor, is a nuclear target of protein kinase A • Gene transcription and transcription factors, from Jacob and Monod until today, histone acetylation and methylation, pre-initiation transcription complex • CREB recruits co-activators, CREBBP, PE300 and CRTC2 • CREB1, FOXO1, PPAR and the glucocorticoid receptor NR4A1 drive the gluconeogenic program • Insulin disables the gluconeogenic program (cytoplasmic sequestration of CRTC2 and FOXO1, phosphorylation and dissociation of CREBBP)	• AKAP79, schematic representation of scaffolding role in signaling complex assembly (ADCY8, PPP3CB, PPP3CA, PRKACA, PRKARIIA, DLG1), role in control of phosphorylation of the AMPA-type glutamate receptor (GRIA1) • bZIP, basic leucine-zipper protein, list of members of the protein family • CREB1, cAMP response-element binding protein, structural composition of bZIP domain bound to cAMP-response element (CRE), phosphorylation sites in KID domain and interaction with CREBBP • GCGR, glucagon receptor, bound to glucagon, predicted structure • Histone-3, methylation and acetylation signatures of a repressed and activated enhancer, promotor and coding region • PIC, transcription pre-initiation complex, schematic representation of proteins • PRKACA, catalytic subunit of protein kinase A, domain architecture, structural composition, conserved residues, position of N-tail with myristate and of C-tail, co- and posttranslational phosphorylation and phosphosite sequence logo of substrates • PRKARIA, regulatory subunit of protein kinase A, domain architecture, molecular structure with or without cAMP, function of pseudo-substrate (RRGAI), dimerization through dimerization domain and CNBA interactions

8. Nuclear receptors Contexts: a. Sperm motility and capacitation b. Mammary gland development c. Consolidating memory (and dealing with pregnancy) Pathways: Nuclear receptor-mediated regulation of gene transcription	• Steroid hormones, everything from domestic animals, the Chinese Pharmacopoeia of 725 AD, to nineteenth and twentieth century personalities in the discovery of steroids • Steroids accumulate in the nucleus, nuclear receptors • Regulation of transcription discovered in giant chromosomes of insect salivary glands • Superfamily of nuclear receptors and recognition of specific promoter enhancer sites (inverted or direct repeat DNA sequences) • Ligand-mediated activation or repression of gene transcription, histone acetylation or de-acetylation • Chaperone (or heat-shock) proteins and the loading of receptors with their ligand • Cooperation with other transcription factors (transrepression or transactivation) • Non-genomic action of nuclear receptors (activation of SRC, interfering with integrin binding, gating of the AMPA-type glutamate receptor) • Paracrine signaling between estrogen and progesterone receptor-bearing epithelial cells and mammary-gland stem cells • Sperm capacitance and motility induced by progesterone in a non-genomic fashion (effect on the CATSPER Ca^{2+} channel) • Glucocorticoid-mediated synapse strengthening • Endocrine disruption in a plastic world (bisphenol A)	• ESR1, estrogen receptor, molecular structure of ligand binding with agonist (DES), detail of coordinating amino acids of the ligand-binding pocket, position of NCOA2 co-activator, molecular structure of dimer of DNA-binding domain, comprising two C4-type Zn^{2+} fingers • Ludwig Fraenkel and the search for progesterone • Nuclear receptors, domain architecture, classification, ligands, molecular composition of ligands • RARA and ESR1, conformational changes of the ligand-binding domains and interaction with NCOR1 or NCOA1, in the presence of an inverse agonist (BMS493), agonists (AM580, DES) or antagonists (BMS614, tamoxifen) • VDR/RXRA, molecular structure of dimer bound to DNA, cryo-electron microscopy-derived structure, crystal structure modeled to cryo-EM, associated ligands 1,25-dihyroxy vitamin D3 and 9-cis retinoic acid

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Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
9. Protein kinase C in oncogenic transformation and cell polarity Contexts: a. Oncogenic transformation b. Cell polarity (spindle orientation in development, migration of astrocytes, and axonal outgrowth) Pathways: a. Classical protein kinase C: - RAF kinase inhibitor, RAF, MAP-kinase pathway, FOS transcription factor, precancer stem-cell program - Jun-kinase-1, JUN transcription factor, precancer stem-cell program b. Atypical protein kinase C: Monomeric GTPase (CDC42), polarity complex (PARD3, PARD6), cell polarity substrates NUMB, LGL, CRB3	• Phorbol ester and the characteristics of inflammation • Protein kinase C family, member of AGC kinases, domain architecture • Classical protein kinase C, role of Ca^{2+} and diacyglycerol (or phorbol ester) in activation of the kinase, displacement of pseudo-substrate sequence by C1 and C2 domains • Atypical protein kinase C, role of AC1 and PB1 domain plus substrate in rendering atypical protein kinases catalytically competent • Protein kinase C anchoring proteins, RACKs, STICKs, and PICKs • Protein kinase C as a potential oncogene, history, phorbol ester and signaling to AP-1 transcription complexes • Role of classical protein kinase C (PRKCA) in facilitating the cancer stem-cell program, role of JNK1 and JUN, and RAF and FOS • Atypical protein kinase C, different types of polarity cues and the localization of PARD-polarity complexes • Discovery of PAR proteins in *Caenorhabditis elegans* (zygote) • Polarity complexes in flies and mice (CDC42, PRKCI or PRKCZ, PARD3, PARD6, GNAI3, GPSM2, INSC, NUMA, and DLG1)	AP-1, activator protein-1, structure composition of JUN and FOS bound to DNA and linked through leucine zipper, different protein combinations (JUN/JUNB, JUN/ATF7, FOS/JUN, FOS/ATP7, and so on), different response elements (TRE, CRE, MARE1, MAREII and ARE) • BP1 domain, molecular structure of type-I, type-II, and type I/II domains, OPCA motif, lysine, heterodimer assembly, and formation of homotypic array • C1A, C1B, and C2 domains, Ca^{2+} binding, membrane binding, role in protein kinase activation • Polarity complexes, composition, membrane anchors (GNAI or CDC42), signaling complex (PARD3, PARD6, atypical PKC), adaptors (GPMS2, NUMA or INSC,GPMS2, DLG1), motor proteins (dynein complex or kinesin complex), and microtubules • Phorbol ester, phorbol-12-myristate-13-acetate, PMA, TPA, molecular composition • PRKCI, atypical protein kinase iota, molecular structure of kinase domain and PB1 domain, potion of AGC tail, and model of activation mechanism (removal of AC1, pseudo-substrate, and PB1, role of substrate in rendering kinase fully competent • Protein kinase C, PKC, classical and novel, family members, molecular structure of domains, priming of the protein kinase through phosphorylation of the AGC tail by mTORC2 (co-translational), and through phosphorylation of the activation segment by PDK1, comparison of conserved phosphorylation sites and phosphosite sequence logo of PRKCA substrates

10. Regulation of cell proliferation by receptor tyrosine protein kinases Context: EGF-mediated activation of gene expression, cell division Pathway EGFR, recruitment of nucleotide exchange factor (SOS/GRB2), activation of monomeric GTPase (RAS), activation of kinase cascade RAF, MEK, MAPK, translocation into the nucleus, regulation of transcription factors	• Receptor tyrosine protein kinases, classification, family • EGF/ERBB receptor, family members, ligands, dimer combinations, intracellular adaptors and effectors • Adaptors and effector proteins of receptor tyrosine kinases, their discovery, SH2 and PTB domains • SH2 and PTB-containing proteins, enzymes, transcription factors, adaptors, docking proteins • *Drosophila* compound eye, *C. elegans* vulval induction, and the elucidation of the Ras–MAP-kinase pathway • EGFR, SOS, RAS, BRAF, MEK1, ERK1 pathway, detail of BRAF dimer • RAS and RAF oncogenes, detail of multiple regulation mechanisms that control RAF • MAP-kinase (ERK) docking sites • MAP-kinase-activated kinases, family tree (MNK, RPS6K, MAPKAPK) • MAPK-mediated phosphorylation of transcription factors, example ELK4 • MNK1-mediated regulation of protein synthesis, phosphorylation of components of the ribosome translation initiation complex • Scaffold for the RAS–MAPK pathway, yeast STE5, mammalian KSR2 • Why are signaling pathways so complicated? • MAP-kinase-related proteins, subfamilies, ERK, JNK, and p38 pathways, family tree • Other branches of EGFR signaling pathways, Ca^{2+}/calmodulin, PI-3-kinase, STAT proteins • Transactivation, from GPCR to EGFR	AKT1, serine/threonine protein kinase, structural composition, conserved residues, substrate-binding site (penetrates into catalytic cleft, comparison with INSR) • BRAF, domains, structural composition, conserved residues, activation mechanism, side-to-side dimerization, oncogenic mutants • EGFR, extracellular and transmembrane segment, domain architecture, structural composition, ligand-mediated conformational changes, CR domain-mediated dimerization • EGFR, intracellular segment, role of LLRRL helix in juxta-membrane segment in membrane binding of receptor monomer and in stabilization of kinase domain in receptor dimer, allosteric regulation through asymmetric dimer formation of two kinase domains • EGFR, kinase domain, detail of regulation of kinase activity through removal of leucine wedge (L858, L861), illustration of how oncogenic L858R mutation removes the inhibitory constraint (increase in kinase activity without need of receptor dimerization) • ELK4 and SRF, DNA-binding domain, SRE enhancer element • INSR, insulin receptor kinase domain, tyrosine protein kinase, structural composition, conserved residues and substrate-binding site (surface-oriented, long tyrosine required to reach catalytic residue, comparison with AKT1) • MAPK1 (ERK2), structural composition, conserved residues, activation mechanism, activation segment phosphorylation

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Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
		• MAP-kinase-related proteins, scheme of three-tier cascades of ERK, p38, and JNK pathways, family tree of MAPK members, activation segment signature • Receptor tyrosine protein kinases, RTK, classification, domain architecture • SH2 domain, selectivity, contextual peptide, and selectivity of phosphotyrosine recognition • SOS1, domain architecture, structural composition, function, tandem-binding sites for RAS.GTP
11. Signal transduction to and from adhesion molecules Context: a. Integrins, cell survival and cell proliferation Pathways: Integrin dimier, paxillin, recruitment of focal adhesion kinase (PTK2), activation by non-receptor tyrosine kinase (SRC), phosphorylation of scaffold protein (BCAR1) - Activation of protein kinase B (AKT) survival pathway	• Three modes of communication (endocrine, paracrine, juxtacrine) • Overview of adhesion molecules, members of the immunoglobulin superfamily (VCAM, ICAM, SIGLEC), junctional adhesion molecule, occludin, claudin, integrin, cadherin, selectin, and cartilage link protein • Highlight of integrin activation, role of talin and its F3 FERM domain • Receptor-mediated integrin activation, RHOA, RAP1, their guanine–nucleotide exchange factors, PI 5-kinase, RASSF5 (Rap-ligand), SKAP1, APBB1IP (Riam), TLN1 (talin) • Focal adhesion complex formation, RHOA, ARHGEF18, PI 5-kinase, PI-4,5-P2, VCL (vinculin), TLN1 (talin), and ACTN1 (α-actinin)	• CD22, Siglec-2, V-set immunoglobulin domain bound to sialic acid, structural composition, intracellular domain ITIM motif • CD44, cartilage link proteins, domain architecture, family members, link domain-bound hyaluronan, molecular structure • CDH, cadherin protein, domain architecture, family members, molecular structure of EC1 and EC2 domains, role of tryptophan, different types of homophilic interactions (cis/trans, involving EC1 and/or EC3) • ITGA, ITGB, integrin proteins, domain architecture, family members, molecular structure (ITGAV, ITGB3), dimer combinations of α- and β-integrin, activation mechanism (switch blade), role of F3 domain of TLN1 in changing the position of the transmembrane segment of β-integrin binding to TLN1

- Recruitment of guanine nucleotide exchange factor (RAPGEF1), monomeric GTPases (RAP1), protein kinase-cassette RAF-MAP-kinase, pathway to cell division cycle - Recruitment of adaptor protein (CRK), recruitment of guanine exchange protein (DOCK1), monomeric GTPase (RAC1), protein kinase (PAK1), protein kinase (JNK1), pathway to cell division cycle	• Focal adhesion signaling complex, PTK2 (focal adhesion kinase), PXN (paxillin), SRC, BCAR1 (Cas) • Focal adhesion signaling complex, PTK2, TLN1 (talin), SOS1, PI 3-kinase, proliferation (RAS–MAPK and STAT pathway) and survival (AKT1) through control of apoptosis • Control of cell division-cycle inhibitor CDKN1B (p27kip) through ubiquitinylation by the SCF^{SKP2} E3–ubiquitin ligase complex (SKP1, SKP2, CUL1 and E2–ubiquitin conjugation enzyme) • Cadherin-mediated activation of PI 3-kinase (survival), microtubule recruitment (vesicle transport, provision of mRNA and proteins) and development of the zonula adherens (actin contractile filaments).	• PTK2, focal adhesion kinase, interaction with BCAR (Cas), PXN (paxillin), domain architecture and phosphorylation sites, other interacting proteins • SCF^{skp2}, E3–ubiquitin ligase complex, schematic representation of structural composition • SEL, selectin proteins, domain architecture, family members, lectin-like domain bound to fucose of SELPLG, structural composition
12. WNT signaling and regulation of cell adhesion and differentiation Contexts: a. Epithelial–mesenchymal transition b. Stem cells Pathways: a. Canonical Wnt b. Noncanonical Wnt	• Dissipation of cell polarity and de-differentiation • Markers of epithelial mesenchymal transition • Wnt family of cytokines, history of discovery (int and Wg), epistatic analysis of *Drosophila* mutants • β-catenin switches TCF from a gene transcription repressor to an activator • Different partners of β-catenin (CDH1, TCF) and the role of AXIN/APC (destruction complex) in elimination of unbound protein • SCF^{BTRC} E3–ubiquitin ligase complex and ubiquitinylation of β-catenin	• CTNNB1, β-catenin armadillo repeats, interaction with fragment of CDH1 (E-cadherin), • CTNNB1, β-catenin armadillo repeats, interaction with fragment of APC (adenomatous polyposis coli), • CTNNB1, β-catenin armadillo repeats, interaction with fragment of AXIN (*Xenopus* axis inhibitory protein) • CTNNB1, β-catenin armadillo repeats, interaction with LEF1 (lymphoid enhancer-binding factor) • CTNNB1, β-catenin N-terminal segment phosphorylation and ubiquitinylation sites

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Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
	• Wnt signaling disables the AXIN/APC destruction complex (role of FZD, LRP, DVL, CSNK1G1, casein kinase) • Wnt signaling, induction of expression of SNAI1, SNAI2 (slug), TWIST1, ZEP1 and suppression of expression of CDH1 (E-cadherin) • Wnt signaling (WNT3 and FDZ7/LPR6) and stem cell self-renewal, stem cell niche of crypts in small intestine, role of R-spondins and LGR5 receptor in boosting Wnt response • Wnt expression and ephrin-B receptors (EphB) • Wnt and planar cell polarity in *Drosophila* and mammalian cells (RHO, ROCK, myosin light chain, actin filament nucleation and organization) • Adenomatous polyposis coli, colon cancer, mutations in CTNNB1, AXIN, and APC	• Domain architecture and domain interactions of components of Wnt pathway (LRP6, DVL1, AXIN, APC, GSK3B, CSNK2A2 (casein kinase), CTNN1B, CDH1) • Domain architecture and domain interactions of TCF7 and TCF7L1 and their association with transcriptional repressor and activator complexes • Epithelium small intestine, villus, crypt of Lieberkühn, enterocyte, Paneth cell, goblet cells, crypt base columnar cell (Lgr5+ stem cell) • Fzd8, Frizzled-8, receptor extracellular segment bound to wnt-8 (xWnt8), structural composition • LEF1, lymphoid enhancer factor, C-terminal segment (HMG-BT) bound to DNA • TCF family members, gene structure and domain architecture
13. Activation of innate immune system: Toll-like receptor-4 and signaling through ubiquitinylation Context: a. Innate immunity (activation of dendritic cell) Pathway: a. TLR4, dimerization by bacterial lipopolysaccharide (LPS), assembly of ubiquitin-mediated signaling complexes	• Sensing microbial universe by pattern-recognition receptors of dendritic cells • Toll and toll-like receptors (TLR), discovery in *Drosophila* mutants, types and ligands • Composition of bacterial cell walls, Gram positive, negative and mycobacteria, lipopolysaccharides acting as a shield and pathogen-associated molecular pattern, which acts as ligand for TLR4 receptor on dendritic cells • LPS-mediated receptor dimerization and assembly of large signaling complexes through assembly of DD domain carrying proteins (MYD88, IRAK4 and IRAK1)	• CBL, E3-ligase, UBE2L3, E2-conjugating enzyme, ubiquitin, substrate complex, structural composition • E3–ubiquitin ligase classes, RING single component (TRAF6, CBL), RING multicomponent (SCF), HECT (HECT, SMURF) and RING-between-RING (RNF31) • Enhanceosome of interferon-β gene (IFNB1), atomic model of IRF3, IRF7, NFKB, JUN and ATF2 bound to their enhancer elements • NKκB/Rel and iκB (ankyrin repeat proteins), family members, domain architecture and proteolytic processing

- Pathway via MYD88, IRAK1, IRAK4, E3–ubiquitin ligase TRAF6, recruitment of TAB2/MAP3K7 and IKBKG/IKBKB/CHUK, activation of NFKB and of JUN/ATF2 - Pathway via TICAM1, TICAM2, E3–ubiquitin ligase TRAF3, recruitment of TANK/IKBKE/TBK1, activation of IRF3	• Signaling complex formation through K63-connected ubiquitin chains, role of TRAF6 E3-ubiquitin ligase complex, binding of TAB2 associated with MAP3K7 (Tak1), and of IKBKG (Nemo) associated with IKBKB (IKKβ) and CHUK (IKKα) • Phosphorylation of NFKBIA (inhibitor of κB), recognition by SCF^{BTRC} E3-ubiquitin ligase complex, K48-ubiquitinylation, destruction by proteasome, nuclear localization of NFKB and induction of gene transcription • MAPK14 (p38a) and MAPK8 (JNK1) activation through MAP3K7 (TAK1), activation of JUN/ATF2 (AP-1 complex) and induction of gene transcription • TLR4-mediated signaling complex assembly comprising TICAM2 (Tram), TICAM1 (Trif) and E3–ubiquitin ligase TRAF3. Ubiquitin-chain formation, binding of TANK, activation of IKBKE and TBK1, leading to phosphorylation of IRF3 followed by nuclear translocation and induction of gene transcription • Feedback mechanisms, holding inflammatory response in check. • Excursion on ubiquitinylation and sumoylation	• TBK1 activation, molecular structure of protein kinase (kinase-+ULD-+SDD domain), conserved residues and phosphorylation of the activation segment, role of TANK binding to the K63–ubiquitin chain in bringing inactive protein kinases together and phosphorylation of the activation segment in trans. • TLR4, toll-like receptor-4, structural composition, leucine-rich repeats, toll-interleukin receptor domain (TIR), death-domain (DD) and interaction with cellular adaptors and effectors • TLR4, receptor dimer-mediated assembly of large signaling complexes through sequential reenforcing DD domain interactions of MYD88 (adaptor), IRAK4 and IRAK1 (kinases), molecular structure and assembly mechanism • Proteasome, model of capture of phosphorylated CTNNB1 by PSMD4 and RAD23A, molecular structure of RAD23A, XPC, ubiquitin-binding domain (UBA) and ubiquitin-like domain (UBL) • Ubiquitinylation process, E1–ubiquitin activation, E2–ubiquitin conjugation, and E3–ubiquitin ligation, isopeptide bond, peptide bond (linear linkage), different linkages, M1, K11, K48, K63
14. Chemokines and traffic of white blood cells Context: Immunity and recruitment of leukocytes to sites of inflammation	• First evidence of extravasation in the tongue of a frog, the account of Augustus Waller (1864) • Bacterial infection (S erysipelas) in treatment from "new growth" and the discovery of tumor necrosis factors • Inflammatory mediators and their sources	• Chemokine receptors, downstream enzymes, their products and biological effects • Chemokines, classification, topology of cysteine bonds • CXCL8, interleukin-8, bound to fragment of CXCR1, receptor, structural composition

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Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
Pathways: a. TNF, TNF-receptor, ubiquitinylation-mediated signaling-complex assembly (TRADD, TRAF2, RIPK1 and the E3–ubiquitin ligase BIRC2), recruitment of TAB2/MAP3K7 (TAK1) and IKBKG/IKBKB/CHUK (IKKα/β), activation of transcription factor NFKB, transcription of inflammatory mediators and enhanced expression of adhesion molecules (among which ICAM1 and VCAM1) b. Chemokine CXCL1 (Gro), CXCR2 receptor, phospholipase-Cβ, phosphatidylinositol-4,5-bisphosphate, inositol-1,4,5-trisphosphate, diacylglycerol, guanine exchange factor RASGRP2, monomeric GTPase RAP1A, activation of intergrins, arresting leukocytes	• TNF receptor (TNFRSF1A), trimerization, assembly of signaling complex through death domains (DD), binding of TRADD, TRAF2, RIPK1 • K63–ubiquitin chain attachment to RIPK1 by the E3–ubiquitin ligase complex BIRC2/UB2D3, binding of IKBKG (Nemo) and TAB2 associated with MAP3K7 (TAK1), IKBKB (IKKβ) and CHUK (IKKα) • Phosphorylation of NFKBIA (inhibitor κB), recognition by $\mathrm{CSF}^{\mathrm{BTRC}}$ followed by its destruction, translocation of NFKB to the nucleus • LUBAC, linear ubiquitin-chain assembly, by E3–ubiquitin ligase RNF31, reenforces signaling complex • Role of E3–ubiquitin ligase PELI3 in preventing apoptotic pathway • Chemokine receptor-mediated activation of phospholipase A2 (PLA2G6), phospholipase C (PLCB2 or PLCG1), phospholipase C (PKD1) and PI3-kinase (PIK3CA), enzyme products and their biological effects • CXCL1 (Gro), CXCR2 and activation of PLCB2, formation of diacylglycerol, activation of RASGRP2 (guanine nucleotide exchange factor), loading of GTP on RAP1A, leading to membrane recruitment of RASSF5, APBB1IP, and talin-mediated activation of integrins, binding to ICAM1, and arresting the cell on the surface of vascular endothelium • Chemokine-mediated migration of leukocytes, role of RHOA and RAC1	• Extravasation, a three-step process, circulation, attachment/rolling and arrest/diapedesis • LUBAC, role of linear ubiquitin chains, M1, in stabilization of signaling complexes, complex of RNF31 (HOIP), RBCK1 (HOIL), SHARPIN • Protrusion and retraction, mode of cell migration, proteins involved, domain architecture, and domain interactions (PI 3-kinase-γ, TIAM, RAC1, GNA13, ARHGEF1, RHOA, ROCK1) • TAB2, adaptor, binding to K63–ubiquitin dimer, structural aspect • RNF31 (HOIP), domain architecture, linear ubiquitin chain formation (M1–G76), RING-between-RING segment of E3–ubiquitin ligase, schematic representation of association with SHARPIN, RBCK1 (Hoil), OTULIN, CYLD, and ubiquitin chains • TNFRSF, members of the tumor necrosis factor receptor superfamily, domain architecture and ligands • TNFRSF1A, TNF-receptor-1, domain architecture, structural composition, schematic representation of receptor associated adaptors and effectors (TRADD, TRAF2, BIRC2, RIPK1) or the inhibitor (BAG4)

15. Activating the adaptive immune system: role of non-receptor tyrosine kinases Context: T-cell activation, T-cell receptor engaged by antigen bound to MHCII Pathways: a. Phospholipase Cγ, phosphatidylinositol-4,5,bisphosphate, diacylglycerol and inositol-1,4,5-trisphosphate, IP$_3$, intracellular free Ca^{2+}, serine/threonine phosphatases (calcineurin), dephosphorylation of transcription factor (NFAT), nuclear translocation b. Protein kinase Cθ, CARD11, adaptor protein BCL10, recruitment of E3–ubiquitin ligase TRAF6, caspase-like protease (MALT1), ubiquitin chain, recruitment TAB2/MAP3K7, IKBKG (Nemo)/IKBKB (IKKβ)/CHUK(IKKα), phosphorylation of inhibitor kappa B, nuclear translocation of transcription factor (NFKB)	• Overview of members of non-receptor protein tyrosine kinases • T-cell receptor, antigen-presenting dendritic cell, MHC class II, CD4, CD28, CD80 • Activation of ZAP70 by LCK, phosphorylation of LAT, recruitment of various effectors, among which phospholipase Cγ (PLCG1) • Production of diacylglycerol and IP$_3$, liberation of Ca^{2+} from intracellular stores, activation of Ca^{2+}/calmodulin-sensitive calcineurin (phosphatase), dephosphorylation of NFAT, nuclear translocation, DNA binding, and gene expression • Activation of atypical protein kinase-θ (PRKCQ), phosphorylation of CARD11 (Carma1), filamentous assembly of BCL10 (CARD domains), binding of MALT1 and TRAF6 leading to ubiquitin-mediated assembly of a large signaling complex, comprising TAB2/MAP3K7, IKBKG (Nemo)/IKBKB (IKKβ)/CHUK(IKKα), phosphorylation of inhibitor kappa B, nuclear translocation of transcription factor NFKB/RELA, DNA binding and gene expression • Interferon-mediated dimerization of IFNAR1 and -2, activation of the non-receptor protein tyrosine kinases TYK2 and JAK1, JAK1-mediated phosphorylation of STAT1 and -2, dimerization, binding of IRF9, nuclear translocation, DNA binding, gene expression • Down-regulation of interferon pathway	• BCL10, adaptor protein with CARD domain, cryo-electromicroscopy-derived evidence of filamentous assembly, formation of a large adaptor structure that recruits numerous MALT1 proteins (caspase-like protease) and these, in turn, recruit TRAF6 proteins (E3–ubiquitin ligase), assembly of a big signaling complex • CARD11 (CARM1), schematic representation of protein unfolding, nucleation site of BCL10 assembly through CARD–CARD interaction • CSK, non-receptor tyrosine kinase, activator kinase of SRC, domain architecture, structural composition, membrane recruitment. and activation through phosphorylated PAG1 (CBP) • IFN, interferons, classification • IFNAR1, IFNAR2, interferon receptors bound to interferon-α (IFNA), molecular structure combined with schematic representation • MHCII, major histocompatibility complex class-II, schematic representation, binding of CD4 • Non-receptor tyrosine protein kinases (NRTK), classification, domain architecture, members acting as oncogenes • SRC, non-receptor tyrosine kinase, domain architecture, structural composition, activation mechanisms, role of the membrane recruitment protein PAG1 (CBP), the upstream protein tyrosine kinase CSK and the tyrosine phosphatase PTPRC (CD45)

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
	• Excursion on non-receptor tyrosine protein kinases	• STAT, signal transducer and activator of transcription, schematic representation of phosphorylation, dimerization, association of IRF9, DNA binding and induction of gene expression • TCR, T-cell receptor, schematic and realistic representation, proximity of CD4 • TCR-mediated activation of NFAT, domain architecture and domain interactions of proteins involved in the signaling pathway
16. Signaling through the insulin receptor: Phosphoinositide 3-kinase and AKT Context: Anabolic action of insulin Pathways: Insulin receptor - Phosphorylation insulin-receptor substrate (IRS1), recruitment phosphatidylinositol 3-kinase, phosphatidyl-3,4,5-trisphosphate, recruitment of kinases PDK1, AKT2	• A brief history of the discovery of insulin • Insulin receptor dimer, change in relative positions of kinase domain (mechanism unknown), activation of kinases through transphosphorylation of activation segment • recruitment of scaffold protein IRS1 and adaptor protein SH2B2 via an phosphotyrosine PTB and SH2 domain respectively • Recruitment of phosphatidylinositol 3-kinases and production of phosphatidylinositol-3,4,5-phosphate • Recruitment of PDK1 and AKT2 through their PH domain, formation of a transient kinase dimer followed by transphosphorylation (in activation segment) and activation of AKT2	• AGC kinases, members, their domain architecture, and common priming and activation mechanism • AKT2, domain architecture, family members, structural composition, hydrophobic motif, activation segment, conserved residues, activation mechanism, role of mTORC2 (priming) and of PDK1 (activation), phosphosite sequence logo of substrates • GSK3B, surface representation of kinase domain, inhibition through phosphorylation of the N-terminal (pseudo-substrate), processive phosphorylation, role in glycogen metabolism • INSR, insulin receptor, extracellular segment, structural composition, α- and β-chains, domain architecture, cleavage sites, and disulphide bonds

- GTPase-activating protein TSC2, monomeric GTPases (RHEB), activation kinase complex (mTORC1), protein kinase (RPS6KB1) and mRNA capping protein (4EBP1), pathway to protein synthesis - GTPase-activating protein (TBC1D4), monomeric GTPase (RAP10), pathway of vesicle transport (glucose transporter supply) - Protein kinase GSK3B, pathway to glycogen synthesis - Recruitment of SH2B2 and guanine nucleotide exchange factor (RAPGEF1), monomeric GTPases (RHOQ), pathway to vesicle docking and fusion proteins (TRIP10, EXOC7)	- Phosphorylation and inhibition of GSK3B, activation of glycogen synthase (GYS) and phosphorylation and inhibition of TSC2 (GTPase-activating protein), accumulation of the monomeric GTPase RHEB in its GTP bound state at the lysosomal membrane, activation of the mTORC1 complex in the presence of RAGA/B bound to GTP and RAGD/D bound to GDP (permissive condition, only occurs in the presence of sufficient amino acids in the lysosome) - mTORC1 phosphorylation and activation of RPS6KB1 and phosphorylation and inhibition of 4EBP1 (removed from EIF4E, bound to the mRNA 5′cap, stimulation of protein synthesis) - 5′-AMP-mediated activation or PRKAA2 (AMPK), with the help of STK11, phosphorylation and activation of TSC2, accumulation of RHEB in GDP-bound state, enhanced autophagy and reduced protein synthesis	- MTORC1, kinase complex, domain architecture, complex composition, MTOR (kinase), RAPTOR (interacting with RRAGA/B), DEPTOR (inhibitor), MLST8 and interaction with FKB12/ rapamycin - MTORC2, kinase complex, domain architecture, complex composition, MTOR (kinase), RICTOR (interacting with PROTOR), DEPTOR (inhibitor), MLST8 - Phosphoinositide metabolism (PI), kinases, phosphatases, and interacting protein domains (ENTH, FYVE, PH, PX, WD-repeat) - PIK3C, PI 3-kinase, phosphatidylinositol 3-kinase, classification, regulatory subunits, upstream receptors (TRK, GPCR/ARRB1/ RALGDS, GPCR/Gβγ) and interacting proteins - PIK3CG, PI3-kinase-γ, structural composition, binding to phospholilpid membrane and phosphatidyl-4,5-bisphosphate, interaction with inhibitors (LY and wortmannin) - Ribosome, translation initiation complex, schematic representation, of components and phosphorylation processes (mRNA, EIF3, EIF4G, EIF4E, IEF2G, EIF4A/4B, tRNA^{met}, RPS6, RPS6KB1, MTORC1 - TSC1, domain architecture, phosphorylation sites - TSC2, domain architecture, phosphorylation sites

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
17. TGFβ and signaling through receptor serine/threonine protein kinases Contexts: a. Cell cycle regulation b. Cell fate decision during development c. Epithelial–mesenchymal transition Pathways: Canonical, SMAD-mediated, pathway Noncanonical pathway: receptor adaptor protein (PARD6), E3–ubiquitin ligase (SMURF1), ubiquitinylation of RHOA, loss of tight junction disassembly	• TGFβ, a matrix-associated cytokine, precursor form • A family or receptors, accessory and pseudo-receptors, ligands and traps • contribution of *Drosophila* and *C. elegans* in the discovery of signaling pathway • Type-1 and -2 receptor, phosphorylation of GS domain, removal of inhibitory wedge, kinase activation, role or ZFYVE9 (SARA) in bringing SMAD to the receptor • SMAD types, receptor regulated, common mediator and inhibitory, phosphorylation of regulated SMAD protein by type-1 receptor, association with common mediator SMAD4, translocation to the nucleus, binding to SMAD-binding element • Association of co-activators (PIN1, YAP1, EP300, CREBP, mediator complex, elongation complex, SWI/SNF • Role of master transcription factors, SPI, POU5F1, MYOD1, in making DNA accessible for SMAD proteins, cell-lineage dependent, cooperative action with other transcription factors (FOXO1, E2F4, STAT3, ATF3, JUN, RBL1) which are controlled by other cytokines (signal integration) • Phosphorylation of linker region determines duration of transcriptional activity, role of kinases CDK8, CDK9 and phosphatase PPM1A and the RNA polymerase-associated phosphatases CTDSP1, CTDSP2 and CTDSPL, regulation of protein half-life by phosphorylation through kinase GSK3B • Mono-ubiquitinylation dissociates SMAD complexes, role of TRIM33/UBE2D3, poly-ubiquitinylation (K48) causes destruction, role of NEDD4L and SMURF1 • Phosphorylation of linker region in cytoplasm prevents nuclear translocation, role of MAP-kinase, and promotes destruction, role of SMURF1	• SMAD, transcription factor, structural composition, MH1 and MH2 domain, DNA binding, schematic representation of phosphorylation and ubiquitinylation sites, composition of trimeric complex • TGFβ, ligand, dimer, cysteine knot, precursor protein LAP-TGFβ • TGFBR, TGFβ receptor type-1 and -2, extracellular segment, domain architecture, structural composition, ligand binding, dimerization • TGFBR, overview of receptor types, pseudo-receptors and accessory receptors, their ligands and traps • TGFBR1, kinase domain, structural composition, GS domain, L45 loop, conserved residues, activation mechanism • TRIM33, bromo- and PHD domain, interaction with acetylated and methylated Histone-3.3

	• Holding the pathway in check, role of inhibitory SMAD proteins, SMAD6 in creating inactive SMAD complexes, SMAD7 in blocking the receptors, and causing their uptake and destruction (SMURF2/SMAD7 complex) • one-morphogenetic protein (BMP, member of the TGFβ family) and fibroblast growth factor (FGF) in the induction of neuro-ectoderm in the *Xenopus laevis* embryo (Spemann organizer activity) • TGFβ and inhibition of cell division cycle progression, SMAD mutations and cancer, role of TGFβ in epithelial–mesenchymal transition	
18. Protein phosphatases Contexts: a. Diabetes and obesity b. Redox regulation and growth factor signaling c. Inflammation d. T-cell activation e. Tumor suppression f. Establishment and maturation of neuronal synapse g. Control of MAP-kinase pathway i. Control of glycogen metabolism	• Overview of superfamily of phosphatases (PTP, PPP, PPM, CTD) • Domain architecture of members of the family of tyrosine phosphatases, both receptor-like (PTPR) and non-receptor (PTPN) • Tyrosine phosphatases, catalytic mechanism, catalytic pocket, and phosphotyrosine specificity • PTPN1 in diabetes and obesity, inhibition of insulin signaling, dephosphorylation of receptor and IRS1, inhibitors of PTPN1 avoid insulin resistance, at the level of glucose transport in muscle and liver as well as at the level of POMC transcription in neurons of the arcuate nucleus involved in the inhibition of appetite (anorexigenic signal)	• DUSP6 (MKP3), dual-specificity phosphatase, surface representation, conserved segments, cartoon representation, interaction with MAPK1 (ERK2) • PPP1CA, serine/threonine phosphatase, structural composition, signature sequence, catalytic pocket, catalytic mechanism, substrate interaction grooves, binding to PPP1R12A (MYPT1), regulatory subunit, determinant in substrate specificity, myosin regulatory light chain, okadaic acid, toxin from dinoflagellates, binding site • PPP5C, serine/threonine phosphatase, structural composition, regulation by TPR domain, TPR-repeats, binding to heat-shock protein HSP90

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
Pathways: a. Multiple	• Redox regulation of PTPN1, H_2O_2-mediated formation of a cyclic sulphonamide that inhibits phosphatase activity leading to amplification of insulin and growth factor signaling • SH2 domain-containing phosphatases, PTPN6 and PTPN11, their regulation through binding of SH2 domains to tyrosine-phosphorylated proteins, the role of PTPN6 in attenuating the inflammatory response, loss-of-function causes severe skin inflammation, role of PTPN11 in boosting RAS–MAP kinase pathway, gain-of-function mutations of the latter phosphatase cause the Noonan and LEOPARD syndrome and are associated with leukemia • receptor-like tyrosine phosphatase TPTRC (CD45), loss-of-function cause severe combined immunodeficiency (SCID), primes LCK for activity, essential for activation of ZAP70 at the T-cell receptor • Receptor-like tyrosine phosphatases PTPRF (LAR), PTPRD, and PTPRS are adhesion molecules, recognize multiple ligands and interaction between the two plays a key role in establishment and subsequent maturation of neuronal synapse, signaling role still undetermined • Dual-specificity phosphatases, domain architecture of family members (classified as subfamily of PTP) • DUSP1 and DUSP6 bind, dephosphorylate and inhibit MAP-kinase, nuclear action of DUSP1 and cytoplasmic action of DUSP6	• PPP3R1 (CnB), PPP3C (can), calcineurin, serine/threonine phosphatase, Ca^{2+} sensitive, molecular structure of complex, dephosphorylation of NFAT; inhibited by FK506 bound to the immunophilin FKBP1A • PTEN, dual-specificity phosphatase, phosphatidylinositol substrate, surface representation, catalytic pocket, conserved segments, domain architecture, cartoon representation of C2 and PTP domain, phosphorylation and ubiquitinylation sites, detail of mutations detected in cancer, regulation of activity by protein kinases (SRC, GSK3B, CSNK2A2 (casein kinase), phospholipid and phosphatidylinositol lipid-binding sites, intrinsic disordered C-tail, binding site for numerous proteins (hub function) • PTPN1 (PTP1B), non-receptor tyrosine phosphatase, structural composition, conserved segments (PTP, Q, and WPD loops), catalytic pocket, phosphotyrosine specificity, catalytic mechanism, redox-regulation of catalytic residue, formation of cyclic sulphonamide • PTPN6 (SHP-1), non-receptor tyrosine phosphatases, structural composition, conserved segments, regulation by SH2 domains • PTPN11 (SHP-2), domain architecture, mutations found in Noonan and LEOPARD syndrome, mutations found in leukemia, consequences for phosphatase activity • PTPRF (LAR), receptor tyrosine phosphatase, surface representation of cytosolic segment, tandem phosphatase domain, conserved segments, dimerization and inhibitory wedge

	• puc, a *Drosophila* dual-specificity phosphatase, controls the activity of bsk (MAP-kinase) and controls epithelial cell movement and elongation in the process of dorsal closure • PTEN, a dual-specificity phosphatase, recognizes phosphatidylinositol phosphates, true tumor suppressor, loss-of-function mutations in Cowden syndrome and in hamartomas development, controls PI3-kinase pathway, regulated by multiple phosphorylation sites, carries an intrinsic disordered C-tail which enables a large range of protein–protein interactions, a single ubiquitin modification causes nuclear translocation, acts in processes that conserves chromosome integrity • Serine/threonine phosphatases, domain architecture of members and of some of their regulatory subunits • Substrate recognition and subcellular localization determined by regulatory subunits • PPP1CA, molecular structure, substrate recognition grooves and catalytic mechanism • PPPC5, phosphatase regulated by intramolecular domain interaction • PPP1CB associated with PPP1R12A (Mypt1), selective for myosin-regulatory light chain, inhibition by okadaic acid (toxin from dinoflagellates)	

Continued

Title/context/main pathway	Subjects	Highlights on molecules, proteins, and personalities
	• PPP1CA bound to PPP1R3A (glycogen targeting in muscle) or PPP1R3B (glycogen targeting in liver), glycogen metabolism, glycogenesis versus glycogenolysis, is discussed in relation to action of protein kinase A (PRKACA) and glycogen synthase kinase (GSK3B) • PPP3C (calcineurin or CnA), control by Ca^{2+}/calmodulin and by PPP3R1 (CnB), dephosphorylation of NFAT, activation of gene transcription in T-cells	
19. Cell fate determination by Notch Contexts: a. Bristle-containing sensory organ development in *Drosophila* wing b. Maintenance of the stem cell niche in the intestinal crypt Pathway: Notch	• Morgan, notched wings of *Drosophila* and the gene theory • Domain architecture of NOTCH2 and its ligands (JAG1, DLL1) • Description of cleavage sites, S1 (furin), S2 (ADAM17), and S3 (γ-secretase complex) in the extracellular membrane-proximal segment, role of S2 and S3 in the signaling mechanism of NOTCH • Cleavage of S3-site liberates the intracellular segment, Nicd, which translocates into the nucleus, binds the transcription factor RBPJ, an event that leads to the loss of transcriptional repressors (NCOR2, HDAC1) and recruitment of transcriptional activators such as EP300 and the mediator complex • Target genes are HES1, HES5, HES7, HEY1, HEY2, and HEYL, transcription factors involved in the determination of cell fate	• HES1, HEY2, helix–loop–helix protein, structure of HLH segment, schematic representation of interaction with TLE4 (groucho) and SIRT1, both histone deacetylases • NOTCH2, domain architecture, structure of EGF-like (class II) repeats, phosphorylation sites, realistic and schematic representation of the extracellular membrane proximal segment comprising the Notch-regulatory region with Lin-12 repeats (LNR) and heterodimerization domain (HD) and containing S1 (furin) S2 (ADAM), S3 (γ-secretase) cleavage sites, highlight of mutations in the extracellular segment that facilitate cleavage and are associated with lymphoblastic leukemia • NOTCH-nicd, RBPK, and MAML1, structure and composition of transcription complex bound to DNA containing a paired response element

- Description of Ncid destruction through phosphorylation of PEST motif (at C-terminal) by CDK8 followed by its recognition by E3–ubiquitin ligase $\mathrm{SCF}^{\mathrm{FBXW7}}$, multi-ubiquitinylation (K48), recognition by proteasome
- Endocytosis is essential for notch signaling in *Drosophila*, both ligand (delta) and receptor (notch), description of proteins involved in uptake of both (itch, nedd4, lap, numb, shibire (dynamin), mib1 and adaptor-protein-2 complex)
- Description of imaginal disks of the 3rd instar larva and the corresponding body parts of the adult fly, focus on the wing imaginal disc
- Description of the mechanoreceptor on thorax and wing, part of the bristle-containing sensory organ, notch signaling in the sensory organ precursor (SOP) gives rise to two cell types (pIIa and pIIb), each with distinct fates (socket and shaft versus sheath, neuron and glia cell)
- Description of intestinal stem cell compartment, expression of NOTCH1 in the cycling crypt-base columnar cells (Lgr5+ stem cells) and expression of DLL4 in the adjacent Paneth cells, NOTCH1-mediated expression of HES1 leads to suppression of ATOH1 (a transcription factor instrumental in the differentiation toward a secretory cell type), one of the mechanisms by which NOTCH1 maintains the stem cell in an undifferentiated (self-renewable) state
- Examples of cross-talk between Notch and with BMP/TGFβ or Wnt-signaling pathways
- NOTCH mutations in lymphoblastic leukemia

CHAPTER

1

Prologue: Signal Transduction from an Historical Perspective

TRANSDUCTION, THE WORD AND ITS MEANING

The expression *signal transduction* first made its mark in the biological literature in the 1970s (Hildebrand, 1977) and appeared as a title word in 1979 (Springer et al., 1979; Koman et al., 1979; Kenny et al., 1979). Physical scientists and electronic engineers had earlier used the term to describe the conversion of energy, or information, from one form into another. For example, a microphone transduces sound waves into electrical signals. The term implies two related activities: one concerns transmission and the other translation of the original signal (a sound wave). Its widespread use in bio-speak was triggered by an important review by Martin Rodbell, published in 1980. He was the first to draw attention to the role of GTP and GTP-binding proteins in metabolic regulation and he deliberately borrowed the term transducer to describe their role in the relay of the receptor signal to the effector (Figure 1-1).

> Alfred G Gilman and Martin Rodbell were awarded the Nobel Prize in 1994 "for their discovery of G-proteins and the role of these proteins in signal transduction in cells."

In the year 2010, 12.6% of all papers using the term *cell* also employed the expression *signal transduction* and 16.6% also employed the expression *signaling* (the American spelling of *signalling*) (*information from PubMed*). The explosion in signal transduction research corresponds with the episode in which it became apparent that oncogenes disrupt ordinary, well-controlled, signaling processes. In particular Ras, the product of the oncogene *ras* leading to the formation of rat sarcoma, and its role in growth factor signaling has been the subject of intense investigation.

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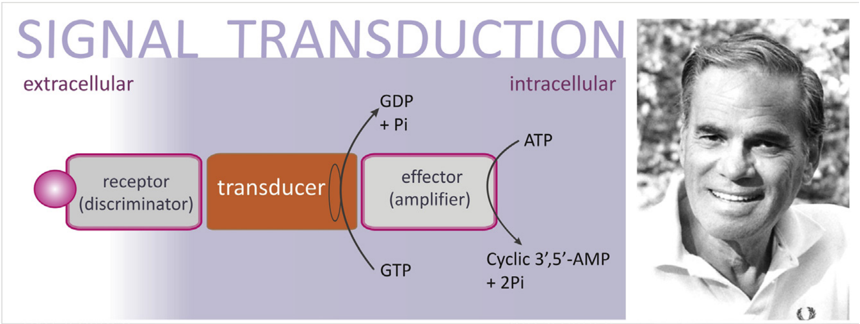


FIGURE 1-1 The transducer. Introduction of the concept of a transducer in the relay of the receptor signal to the effector. A transducing GTP-binding protein relays the receptor signal, we speak of signal transduction. *Adapted from* Rodbell (1980). *Image of Rodbell from* http://www.nobelprize.org/.

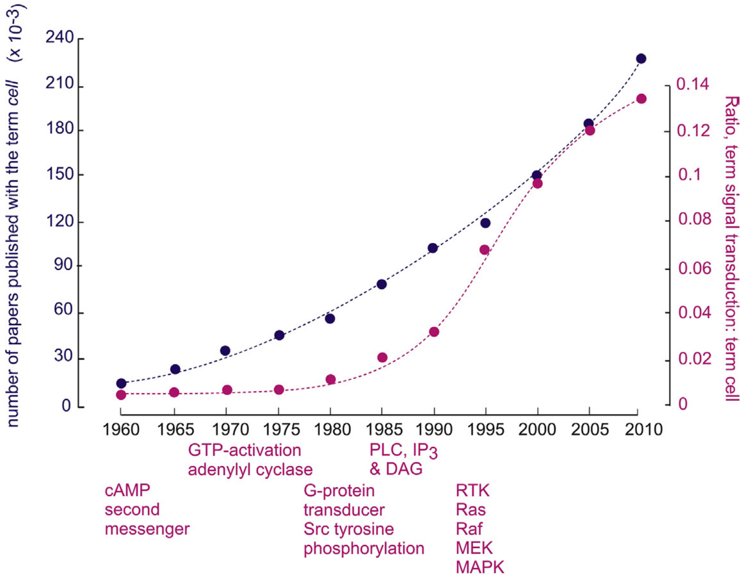


FIGURE 1-2 Occurrence of the term *signal transduction*. The left-hand axis records all papers using the term *cell* traced through the PubMed database. The right-hand axis records the proportion of papers using the term *cell* that also use the term *signal transduction*. Major discoveries that have boosted signal transduction research are shown underneath the time axis.

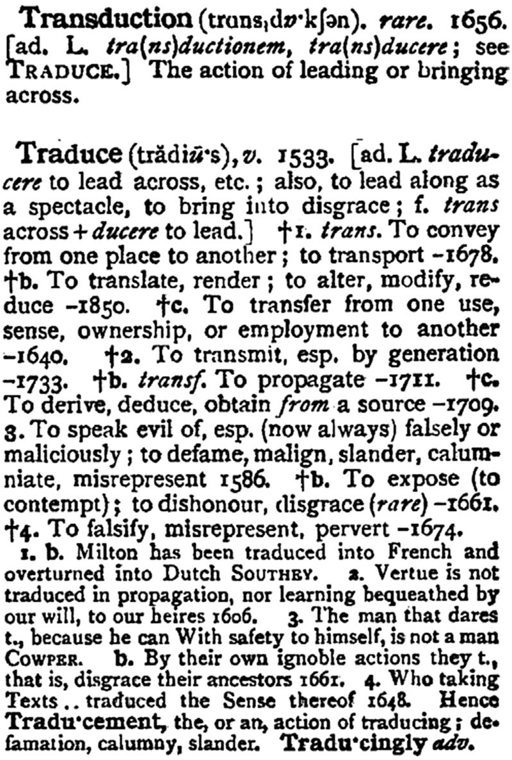
It occurs that signaling mechanisms are an important research domain in biological sciences (Figure 1-2).

Below follows a description of personalities and experiments, during the transition from the nineteenth to the twentieth century, that have paved the way to our current understanding of how different parts of the body communicate with one another (hormones, neurotransmitters, growth factors). Naturally, major discoveries gave rise to controversy (as they challenged hegemony of ideas and personalities) and, as a consequence, a good dose of (persistent) anxiety for those who deviated from the trodden path. Our account also shows that in many instances, scientists did not really know what to look for and thus what to expect from their experiments. Only through the course of their experimentations did they develop a sense of direction and importantly, understanding. Had they been confronted with current grant application forms, the section "expected outcome" would be manifested by its blankness.

Transduction entries in the *Oxford English Dictionary* (Figures 1-3 and 1-4)

IRRITABILITY, A VITAL PHENOMENON

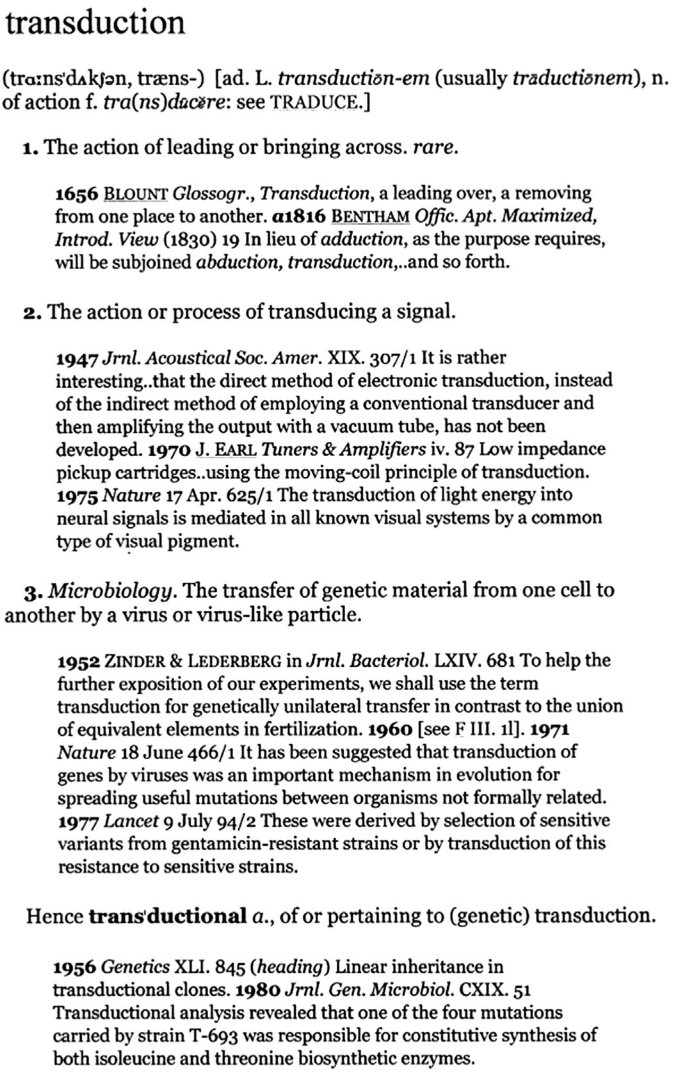
The origin of life is often considered as fundamentally a problem of the origin of template replication. However, replication itself is not



Transduction (trɒns,dɒ·kʃən). *rare.* 1656. [ad. L. *tra(ns)ductionem*, *tra(ns)ducere*; see TRADUCE.] The action of leading or bringing across.

Traduce (trădiū·s), *v.* 1533. [ad. L. *traducere* to lead across, etc.; also, to lead along as a spectacle, to bring into disgrace; f. *trans* across + *ducere* to lead.] †1. *trans.* To convey from one place to another; to transport -1678. †b. To translate, render; to alter, modify, reduce -1850. †c. To transfer from one use, sense, ownership, or employment to another -1640. †2. To transmit, esp. by generation -1733. †b. *transf.* To propagate -1711. †c. To derive, deduce, obtain *from* a source -1709. 3. To speak evil of, esp. (now always) falsely or maliciously; to defame, malign, slander, calumniate, misrepresent 1586. †b. To expose (to contempt); to dishonour, disgrace (*rare*) -1661. †4. To falsify, misrepresent, pervert -1674.
1. b. Milton has been traduced into French and overturned into Dutch SOUTHEY. 2. Vertue is not traduced in propagation, nor learning bequeathed by our will, to our heires 1606. 3. The man that dares t., because he can With safety to himself, is not a man COWPER. b. By their own ignoble actions they t., that is, disgrace their ancestors 1661. 4. Who taking Texts .. traduced the Sense thereof 1648. Hence **Tradu·cement**, the, or an, action of traducing; defamation, calumny, slander. **Tradu·cingly** *adv.*

FIGURE 1-3 From the Shorter *Oxford English Dictionary* (3rd edition, 1994, with corrections 1977, © Oxford University Press).



transduction

(trɑːnsˈdʌkʃən, træns-) [ad. L. *transductiōn-em* (usually *trāductiōnem*), n. of action f. *tra*(*ns*)*dūcĕre*: see TRADUCE.]

1. The action of leading or bringing across. *rare*.

1656 BLOUNT *Glossogr.*, *Transduction*, a leading over, a removing from one place to another. **a1816** BENTHAM *Offic. Apt. Maximized, Introd. View* (1830) 19 In lieu of *adduction*, as the purpose requires, will be subjoined *abduction, transduction*,..and so forth.

2. The action or process of transducing a signal.

1947 *Jrnl. Acoustical Soc. Amer.* XIX. 307/1 It is rather interesting..that the direct method of electronic transduction, instead of the indirect method of employing a conventional transducer and then amplifying the output with a vacuum tube, has not been developed. **1970** J. EARL *Tuners & Amplifiers* iv. 87 Low impedance pickup cartridges..using the moving-coil principle of transduction. **1975** *Nature* 17 Apr. 625/1 The transduction of light energy into neural signals is mediated in all known visual systems by a common type of visual pigment.

3. *Microbiology*. The transfer of genetic material from one cell to another by a virus or virus-like particle.

1952 ZINDER & LEDERBERG in *Jrnl. Bacteriol.* LXIV. 681 To help the further exposition of our experiments, we shall use the term transduction for genetically unilateral transfer in contrast to the union of equivalent elements in fertilization. **1960** [see F III. 1l]. **1971** *Nature* 18 June 466/1 It has been suggested that transduction of genes by viruses was an important mechanism in evolution for spreading useful mutations between organisms not formally related. **1977** *Lancet* 9 July 94/2 These were derived by selection of sensitive variants from gentamicin-resistant strains or by transduction of this resistance to sensitive strains.

Hence **transˈductional** *a.*, of or pertaining to (genetic) transduction.

1956 *Genetics* XLI. 845 (*heading*) Linear inheritance in transductional clones. **1980** *Jrnl. Gen. Microbiol.* CXIX. 51 Transductional analysis revealed that one of the four mutations carried by strain T-693 was responsible for constitutive synthesis of both isoleucine and threonine biosynthetic enzymes.

FIGURE 1-4 From the *Oxford English Dictionary* (2nd edition, 2008 © Oxford University Press online).

sufficient, metabolism was another important property right from the beginning (Dyson, 1999). According to Thomas Henry Huxley (Figure 1-5), a third essential element for the living comprises a stimulus-response system. Ultimately, this system became the basis of how organisms respond to the environment, and how parts of an organism (or whole organisms) communicate with one another. In his lecture on

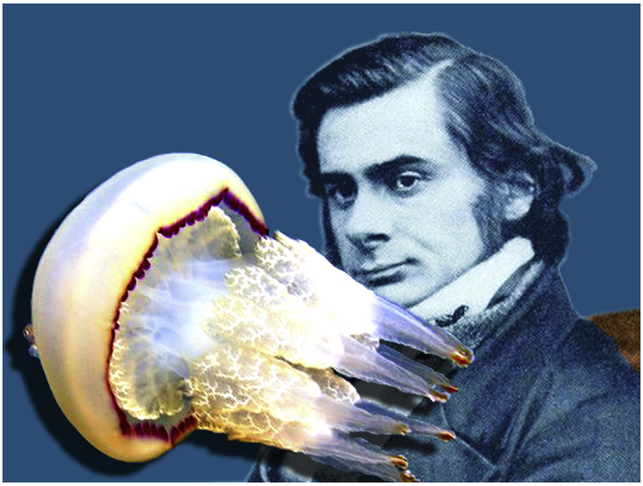


FIGURE 1-5 Irritable medusa and Thomas Henry Huxley. On the left, *Rhizostoma pulmo*, one of the species studied by Huxley. On the right, Thomas Huxley around 1857, 10 years after his journey on the HMS Rattlesnake. *Image of* Rhizostoma pulmo *from Willy Couard (*www.souslemers.fr*) and image of Huxley from the Royal Society (*www.darwininlondon.com*).*

"the physical basis of life" (*Edinburgh, on the evening of Sunday November 8, 1868*), Huxley argues that all living things, ranging from Amoebae to Homo sapiens, are substantially similar in kind with respect to elementary functions and substance (which he named "protoplasm"). Irritability, Huxley's description of stimulus-response coupling, takes an essential place among the list of "vital phenomena."

> In physiological language this means that all the multifarious and complicated activities of man are comprehensible under three categories: either they are immediately directed towards the maintenance and development of the body ("metabolism"), or they effect transitory changes in the relative positions of parts of the body ("stimulus-response"), or they tend towards the continuance of the species ("template replication"). Even those manifestations of intellect, of feeling, and of will, which we rightly name the higher faculties, are not excluded from this classification, inasmuch as to everyone but the subject of them, they are known only as transitory changes in the relative positions of parts of the body. Speech, gesture, and every other form of human action are, in the long run, resolvable into muscular contraction, and muscular contraction is but a transitory change in the relative positions of the parts of a muscle. But the scheme which is large enough to embrace the activities of the highest form of life, covers all those of the lower creatures. The lowest plant, or animalcule, feeds, grows, and reproduces its kind. In addition, all animals manifest those transitory changes of form which we class under irritability and contractility; and, it is more than probable, that when the vegetable world is thoroughly explored, we shall find all plants in possession of the same powers, at one time or other of their existence.

IRRITABILITY

Perhaps it were the medusa that he studied during his voyage on the HMS Rattlesnake (around 1846), that led Huxley to employ the word "irritability" and "contractility" (rather than stimulus-response coupling). Describing the stomach, situated under the disc of *Rhizostoma* he writes: "From this 'common canal' a series of parallel diverticula are given off at regular intervals, and run to the edge of the branch, where they terminate by rounded oblique openings. It is not always easy to see these apertures, but I have repeatedly satisfied myself of their presence by passing a needle or other delicate body into them. The difficulty in seeing the openings arises in great measure from the presence of a membrane which surrounds and overlaps them, and being very irritable, contracts over them on being touched" (Huxley, 1849).

CELLULAR THEORY AND PROTOPLASM

The initial microscopic observations of cellular structures by Robert Hooke (around 1653) and Anthony van Leeuwenhoek (around 1682) obtained full recognition when in 1832 the German botanist Matthias Schleiden proclaimed that cells were the elementary structures of plants. A few years later, Theodore Schwann showed that the animal tissues were also made up of cells, and that they owed their beginning and development to the activity of cell elements; thus originated the "cellular theory." Until 1888, the composition of the brain remained, however, an enigma. In that year Santiago Ramon y Cajal, using Camillo Golgi's silver nitrate impregnation staining, convincingly demonstrated that even the soft, near homogeneous, gray matter was made up of distinctive structures, the nerve cells.

In his "Manual of Physiology" (1889) Gerald Yeo, from King's College, describes the characteristics of the cell as follows: "The first idea which was conveyed by the term cell varied much from that which we now accept as a proper definition of such an organic unit. Fully developed vegetable cells being the first discovered were taken as the type of all. The main characteristics of these may be briefly summed up. First, a membranous sac called the cell wall, generally very well defined, and, secondly, within the cell wall various cell contents. Among the more conspicuous of the latter may be mentioned (1) a soft, clear, jelly-like substance called protoplasm, in which lies a nucleus, and (2) certain cavities called vacuoles, which are filled with a clear fluid or cell sap" (Yeo, 1889).

At that time, the English scientific establishment was still closely tied to the Church of England, while science was part of "natural theology." Scientists merely played the role of explaining the book of Genesis in biological terms. Both Darwin's findings about the origin of species (*On the origin of species*, published in 1859) and Huxley's findings of common "protoplasm" strongly conflicted with the beliefs that species were unchanging parts of a designed hierarchy and that humans were unique, unrelated to other animals (or any species). Huxley, therefore, in a humorous way, cautions his audience about possible conflict with religious beliefs: "But I bid you beware that, in accepting these conclusions, you are placing your feet on the first rung of a ladder which, in most people's estimation, is the reverse of Jacob's, and leads to the antipodes of heaven. It may seem a small thing to admit that the dull vital actions of a fungus, or a foraminifer (protozoa), are the properties of their protoplasm, and are the direct results of the nature of the matter of which they are composed. But if, as I have endeavoured to prove to you, their protoplasm is essentially identical with, and most readily converted into, that of any animal, I can discover no logical halting-place between the admission that such is the case, and the further concession that all vital action may, with equal propriety, be said to be the result of the molecular forces of the protoplasm which displays it."

Quite astonishingly, he ends the lecture as a molecular scientist *avant la lettre*, by interpreting the "transitory changes in the relative positions of parts of the body" as molecular changes! He writes: "And if so, it must be true, in the same sense and to the same extent, that the thoughts to which I am now giving utterance, and your thoughts regarding them, are the expression of molecular changes in that matter of life which is the source of our other vital phenomena" (Lecture source at: http://human-nature.com/darwin/huxley/chap6.html).

And indeed, we now know that as you have read these lines, thousands of GDPs have been replaced by GTPs, rapidly followed by their hydrolysis, thousands of phosphates have been passed from ATPs to proteins and back again (but indirectly), stretches of DNA have been methylated and certain histones have been acetylated. All this activity in the hope that you have not only provided meaning to these words, but also will remember some of them!

MOLECULE

The word molecule is derived from *molecula*, diminutive of the Latin word *mole* and, therefore, translated as "small mass." Although the term was used widely at the onset of the nineteenth century, it was not until 1873, well after Huxley's lecture, that, the Scottish physicist James Clerk Maxwell published an article in *Nature* in which he defines the term molecule as we still handle it today "An atom is a body which cannot be cut in two; a **molecule** is the smallest possible portion of a particular substance."

FLESH AND GRASS, GERRIT MULDER AND JOHANNES BRAHMS

That the composition of plant and man is essentially not that different was already revealed by the Dutch chemist/physician Gerrit Mulder in 1835, working in Rotterdam, when he showed that egg white is composed of a base ("wortelstof") comprising carbon, hydrogen, and nitrogen to which is added a pinch of phosphor and sulfur. This base (essentially an amino acid) was present both in animals and plants. According to his own words "I am the first to show that the flesh is present in the bread and the cheese in the grass" (in old Dutch: "dat het vleesch in het brood aanwezig is en de kaas in het gras"). On advice of Jöns Jacob Berzelius (Stockholm, Sweden), he named the omnipresent base-substance "protein." Derived from the Greek *proteios* (or *protos*), meaning "first" (in this context, "most abundant" or "most eminent").

In the same year as Huxley lectures about the physical basis of life, Brahms finishes his "Ein Deutsches Requiem" with a second movement entitled "For all flesh is as grass" (in German, "Denn alles Fleish, es ist wie gras"). Here, however, the oratorio wishes not to reveal the similarity between man and plants in a chemical sense, quite the opposite, it wishes to accentuate the supremacy of the Divine by quoting Peter: "man is like grass, and like grass its glory withers and its flowers fall away, but the Lord endures forever."

About 140 years later, in his book *Creation: life and how to make it*, Steve Grand, the father of the android robot Lucy and of the tribe of computer creatures called Norns, goes as far as to declare that life "is made not of atoms, it is merely built out of them. What life is actually 'made of' is cycles of cause and effect, loops of casual flow. These phenomena are as real as atoms – perhaps even more real" (Grand, 2001). To stress that physical matter is not essential for our lives he gives the example of long-range memories: "by now I hope you have thought of an experience from your childhood. Something you remember clearly, something you can see, feel, maybe even smell, as if you were really there. After all, you really where there at the time, weren't you? How else would you remember it? But here is the bombshell: you weren't there. Not a single atom that is in your body today was there when that event took place. Every bit of you has been replaced many times over (which is why you eat of course)."

> Matter flows from place to place and momentarily comes together to be you. Whatever you are, therefore, you are not the stuff of which you are made. To see yourself as persistent phenomenon, when the substrate from which you are made is in constant flux, is to begin to understand life, and more than just life.

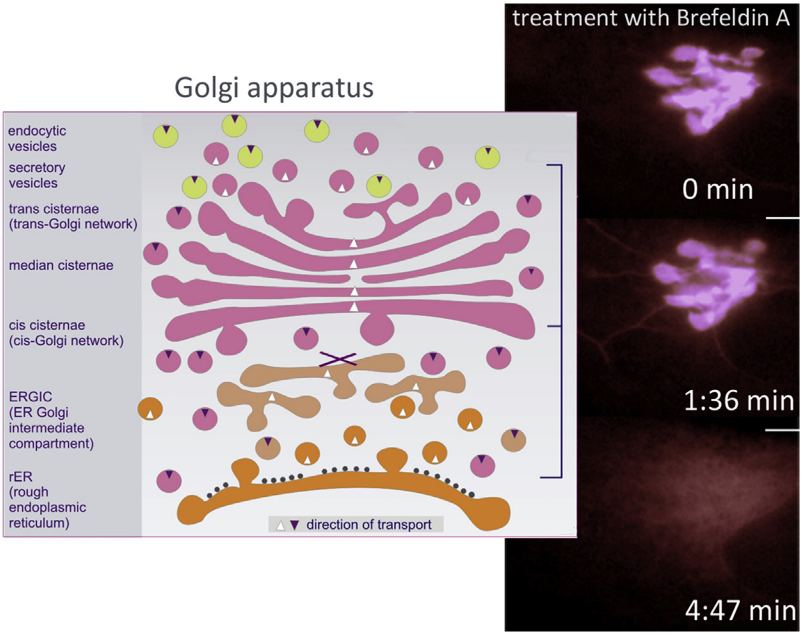

Golgi apparatus
endocytic vesicles
secretory vesicles
trans cisternae (trans-Golgi network)
median cisternae
cis cisternae (cis-Golgi network)
ERGIC (ER Golgi intermediate compartment)
rER (rough endoplasmic reticulum)
△▼ direction of transport
treatment with Brefeldin A
0 min
1:36 min
4:47 min

FIGURE 1-6 Flux and persistence of the Golgi apparatus. The relationship between "flux" and "persistence" as revealed by blocking the maturation of ERGIC vesicles (by brefeldin A) into cis-Golgi cisternae. The Golgi apparatus disappears within 5 min of treatment. *Image courtesy of J. Lippincott-Schwartz, Bethesda, USA.*

If this is all too esoteric, the example of the Golgi apparatus may perhaps be revealing to explain how "persistence" and "flux" relate to each other. As you probably will have seen or even studied in numerous electron micrographs, the Golgi comprises stacks of four to eight membrane-enclosed discs, the so-called cisternae, surrounded by numerous small transport vesicles. That is the static picture. However, if you block the transition of ERGIC to the cis-Golgi cisternae (by brefeldin A or dominant negative Arf1), a near-immediate dissolution of the organelle ensues and it only takes 5 min to eliminate the entire Golgi from the cell (Figure 1-6) (Ward et al., 2001). The electron-micrograph stills are deceiving because the Golgi is not a stable but a steady-state organelle: it exists as a consequence of a continuous flow of vesicles, trafficking both in an antro- and retrograde fashion (up and down), undergoing fusion (giving rise to cisternae) and fission.

In this book, we write abundantly about flux, of how cells constantly create new networks through subtle changes in protein shapes and protein composition (posttranslational modifications), leading to new protein interactions, altered subcellular localization or altered

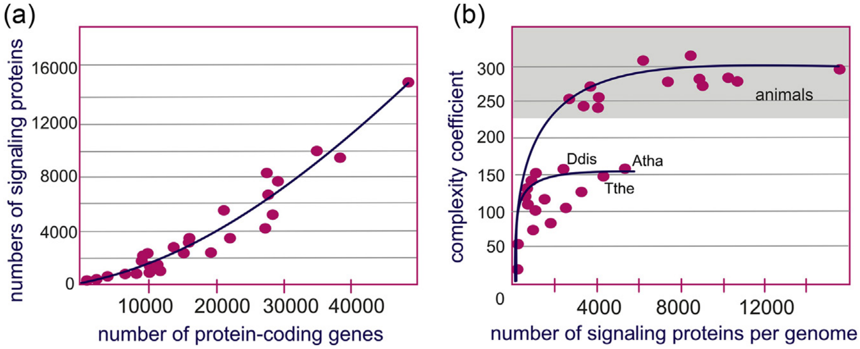


FIGURE 1-7 Signaling matters (a) Nonlinear scaling of the total number of signaling proteins in eukaryotes plotted against the number of protein-coding genes (representing the proteome). (b) Complexity quotient plot for signaling proteins. The complexity quotient for an organism is defined as the product of two values: the number of different types of domains that co-occurs in signaling proteins and the average number of domains detected in these proteins. The complexity quotient is plotted against the total number of signaling proteins in given organisms. Atha, *Arabidopsis thaliana* (plantae); Ddis, *Dictyostelium discoideum* (amoebozoa); Tthe, *Tetrahymena thermophila* (protozoa). *Image adapted from Anantharaman et al. (2007).*

activity or any combination of these, and forming the basis of cellular persistence.

Changes in the wiring of proteins, this time through addition of new protein-interaction domains at the genomic level, also underlie the evolutionary process; coupling stimuli to different (new) responses. Analysis of whole genomes from different organisms revealed two important facts in this respect. First, when the genome (protein-coding genes) of the organism increases in size, the proportion of genes dedicated to cellular signaling increases as well (Figure 1-7(a)), from roughly 20–30%. Second, more detailed analysis of the domain architecture of the proteins involved in signaling reveals that, on average, animals have much more complex domain architectures than plants or protists (Figure 1-7(b)). What this means is that animals not only have bigger genomes, with many more signaling proteins, these proteins also can branch to a larger number of other signaling components. Together this leads to significantly denser signaling networks. Lastly, comparison between genomes of protists points to a greater variety in regulatory proteins than expected from the relatedness of the lineages (Anantharaman et al., 2007). This is taken to mean that signaling proteins determine eukaryotic diversity, that is, differences in signaling networks, connecting receptors with gene transcription, signify differences in species; again, signaling matters.

PROTISTS

They are a diverse group of eukaryotic microorganisms. Historically, protists were treated as the kingdom Protista, which includes mostly unicellular organisms that do not fit into the other kingdoms, but this group is contested in modern taxonomy. Instead, it is better regarded as a loose grouping of 30 or 40 disparate phyla with diverse combinations of trophic modes (they rely on organic nutrition), mechanisms of motility, cell coverings, and life cycles. The protists do not have much in common besides a relatively simple organization—either they are unicellular or they are multicellular but without specialized tissues. This simple cellular organization distinguishes the protists from other eukaryotes, such as fungi, plants, and animals. Protists have two subkingdoms: algae and protozoa.

Source: Wikipedia

In his book *Wetware*, Denis Bray, takes the point that cells are computing devices, clever cells employing algorithms with Boolean logic (AND, NOT, OR), able to store information, memorize current or even past conditions, and being able to take decisions in a calculated manner (Bray, 2009). Cells calculate not through electric networks but, as mentioned above, through molecular networks, implying all major cellular components (lipids, sugars, proteins, and nucleotides). Each of these components acts as a switch as they exist in different shapes (allostery) or compositions or both. Each component may thus act as a binary switch but collectively they seem to give rise to analogous responses. Binary sets that do not act as two-valued logic (on/off) are said to follow the path of *fuzzy logic* (with truth values ranging in degree between zero and one) but here we start to maneuver on very thin ice. The book rightly makes the point that currently we are not able to translate the innumerable cellular processes into a comprehensive system (or comprehensive systems), which would allow us to predict or calculate cellular responses in silico. There are still too many unknown variables (like missing proteins, unknown quantities and enzymatic rates, undiscovered catalytic and allosteric functions) to create a comprehensive signaling system for animal cells. In modern jargon, the mammalian *interactome* is far from complete. Moreover, to return to Steve Grand, we describe signaling processes (fluxes) as ordered linear events, with hierarchal order—starting at the membrane and ending in the nucleus—but you should be aware that they are not. All processes described are parts of a web where the prime cause of any particular circumstance is under the influence of the effect. Glands secrete hormones (cause) that affect cellular activity (effect), and altered cellular activity (cause) in turn affects secretion of the hormone by the gland (effect), etc. In the words of Steve Grand "every cause is the effect of at least one other cause" and "control is therefore as much an effect as a cause." Despite its immense potential in helping to understand cellular functioning, we will only briefly deal with the subject of "systems biology" because we feel that it does not yet facilitate the understanding for those who enter the field of signal transduction.

PROTOENDOCRINOLOGISTS

Despite excellent anatomical descriptions, almost nothing was known of the functions of the various organs, which constitute the endocrine system (glands) until the last decade of the nineteenth century. Indeed, in the standard textbook of the period (*Foster's Textbook of Physiology*, 3 volumes and more than 1200 pages), consideration of the thyroid, the pituitary, the adrenals ("suprarenal bodies"), and the thymus is confined to a brief chapter of less than 10 pages, having the title "On some structures and processes of an obscure nature."

In writing these paragraphs, we have relied heavily on the work of Victor Medvei (1993), Michael Aminoff (1993), and Horace Davenport (1991).

The initial impetus prompting the systematic investigations that led to the discovery of the hormones can be ascribed to a series of papers that were much misunderstood (Medvei, 1993; Aminoff, 1993; Davenport, 1991). However, here we are confronted with the work of Charles-Edouard Brown-Séquard, the successor of Claude Bernard at the Collège de France and a member of leading scientific academies also in England and the USA. He had held professorial appointments at both Harvard and Virginia; in London he was appointed physician at the National Hospital for the Paralysed and Epileptic (now the National Hospital for Neurology and Neurosurgery). He was an associate of Charles Darwin and Thomas Huxley. He wrote over 500 papers relating to many diverse fields such as the physiology of the nervous system, the heart, blood, muscles and skin, the mechanism of vision, and much more. He was an outstanding experimentalist making fundamental contributions. Starting with his doctoral thesis, he described the course of motor and sensory fibers in the spinal cord, a field to which he returned many times. He was in constant demand as lecturer, teacher, and physician on both sides of the Atlantic, crossing the ocean on more than 60 occasions. Of direct relevance to us must be his demonstration that the adrenal glands are essential to life.

In view of all this, it is curious that Brown-Séquard is now all but forgotten (Figure 1-8). On the rare occasions when he is recalled, it is generally

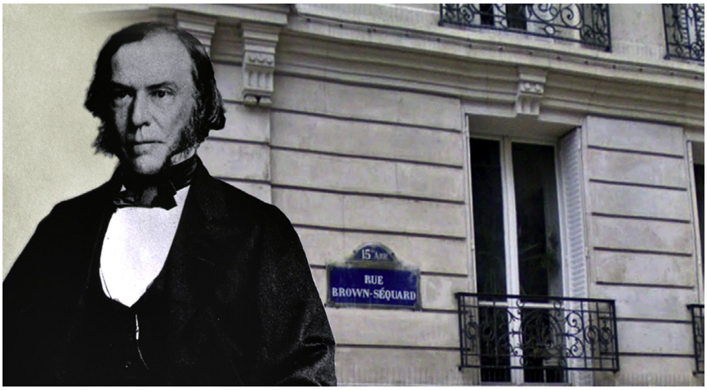


FIGURE 1-8 Brown-Séquard in the street named after him in Paris (15ème). *Source: Google Maps and Wikipedia (Charles-Edouard Brown-Séquard).*

in connection with a series of brief reports, published in 1889, in which he described the self-administration by injection, of testicular extracts, which he considered had the effect of reinforcing his bodily functions.

Some brief quotations from his paper in the Lancet must suffice (Brown-Séquard, 1889a).

> I am seventy-two years old. My general strength, which has been considerable, has notably and gradually diminished during the last ten or twelve years. Before May 15th last, I was so weak that I was always compelled to sit down after about half an hour's work in the laboratory. Even when I remained seated all the time I used to come out of it quite exhausted after three or four hours of experimental labour…
>
> The day after the first subcutaneous injection, and still more after the two succeeding ones, a radical change took place in me, and I had ample reason to say and to write that I had regained at least all the strength I possessed a good many years ago…
>
> My limbs, tested with a dynamometer for a week before my trial and during the month following the first injection, showed a decided gain of strength…
>
> I have measured comparatively, before and after the first injection, the jet of urine in similar circumstances - i.e., after a meal in which I had taken food and drink of the same kind and in similar quantity. The average length of the jet during the ten days that preceded the first injection was inferior by at least one quarter of what it came to be during the twenty following days. It is therefore quite evident that the power of the spinal cord over the bladder was considerably increased…
>
> I will simply say that after the first ten days of my experiments I have had a greater improvement with regard to the expulsion of faecal matters than in any other function. In fact a radical change took place, and even on days of great constipation the power I long ago possessed had returned…

Finally, in a footnote:

> It may be well to add that there are good reasons to think that subcutaneous injections of a fluid obtained by crushing ovaries just extracted from young or adult animals and mixed with a certain amount of water, would act on older women in a manner analogous to that of the solution extracted from the testicles injected into old men.

SECONDE NOTE SUR LES. EFFETS PRODUITS CHEZ L'HOMME PAR DES.INJECTIONS SOUS-CUTANÉES D'UN LIQUIDE RETIRÉE DES TESTICULES FRAIS DE COBAYE ET DE CHIEN.

par M. BROWN-SÉQUARD. (*Communication faite le 15 juin*)

Non seulement il n'y a pas à s'étonner que l'introduction dans le sang de principes provenant de testicules de jeunes animaux soit suivie d'une augmentation de vigueur, mais encore on devait s'attendre à obtenir ce résultat. En effet, tout montre que la puissance de la moelle, épinière et aussi, mais à un moindre degré, celle du cerveau, à chez l'homme adulte ou vieux, des fluctuations liées à l'activité fonctionnelle des testicules. Aux faits que j'ai mentionnés, à cette égard, dans la séance du 1re juin, je crois devoir ajouter que les particularités suivantes ont été observées un très grand nombre de fois, pendant plusieurs années, chez deux individus âgés de quarante-cinq à cinquante, ans. Sur mon conseil, chaque fois qu'ils avaient à exécuter un grand travail physique ou intellectuel, ils se mettaient dans un état de vive excitation sexuelle, en évitant cependant toute éjaculation spermatique. Les glandes testiculaires acquéraient alors temporairement une grande activité fonctionnelle, qui était bientôt suivie de l'augmentation désirée dans la puissance des centers nerveux.

Possibly Brown-Séquard should be regarded as the father of hormone replacement therapy (HRT) (Spiegel, 1996), but he was certainly not the inventor of organotherapy, the attempt to cure human disease by the introduction of glandular extracts from other animals. Indeed, Gaius Plinius Secundus (known as Pliny the Elder, 23–79 CE) recorded his contempt of the Greeks who used human organs therapeutically, although he did recommend the use of animal tissues, in particular, that the testicles of animals should be eaten in order to cure impotence or to improve sexual function in men, and that the genitalia of a female hare should be eaten by women in order to achieve pregnancy (Medvei, 1993).

Fritz Spiegel (1996) reminds us that in Chinese medicine the effect of HRT is allegedly achieved by the administration of rhinoceros horn

Ever mindful of the possibility of autosuggestion, Brown-Séquard pleaded that others should examine his claims and to consider them with objectivity. What a hope! But if any doubts remained, he went on record saying (Brown-Séquard, 1889b):

> Not only is there no occasion for surprise that the introduction into the blood of principles derived from the testicles of young animals should be followed by an increase in vigour: such a result should indeed have been foretold. For in point of fact all the evidence shows that in the adult or elderly male the power of the spinal cord and also, albeit to a lesser degree, that of the brain is subject to variations linked to the functional activity of the testicles. In addition to the facts mentioned in this regard at the meeting of June 1st, I believe I should add that the following particularities have been observed on many occasions during the course of several years in the case of two individuals aged between 45 and 50… On my advice, each time they had to undertake a great physical or intellectual task they put themselves into a state of sexual excitement, while nevertheless avoiding ejaculation. The testicles became substantially active for a time and this was soon followed by the desired increase in the power of the nervous system.

Pentacle: a five-pointed star, of supposed supernatural significance: see http://www.witchvox.com/basics/pentacle.html.

And **respectable** it remained, anyway in France, where in the 1920s Serge Voronoff was treating elderly gentlemen by implanting sections of chimpanzee or baboon testicles, the so-called "monkey glands" (Borrell, 1976). R V Short (Murray, 1891) speculates that his patients may have been the first humans to become infected by HIV-1. Were it not for the fact that they remained impotent, the disease might have exploded on a world even less prepared than it was at the century's end. One might reasonably imagine that an AIDS epidemic originating at that time could have changed the course of twentieth century history.

News travels fast and news of this sort travels faster still. Within weeks this was the subject of an editorial paragraph in the British Medical Journal under the heading The Pentacle of Rejuvenescence (Editorial annotation, 1889).

Many influential members of the English scientific establishment, however, strongly renounced his experiments, condemning them as "disgusting" and acts of "self abuse." Despite this, like it or not, many physicians, aware of Brown-Séquard's publications, were eager to test the possibilities of applying various organ extracts in their practice. Within a year or two, organotherapy was becoming respectable (Editorial annotation, 1925; Short, 1999). In a letter to his colleague Jacques Arsène d'Arsonval (quoted by Borrell (1976)), Brown-Séquard wrote that "the thing" was "in the air." What made it especially so may have been George Redmayne Murray's report (Murray, 1891) on the treatment of myxedema. He described a patient who was treated with regular injections of sheep thyroid extract and went on to survive for further 28 years.

Henry Hallett Dale's description of the discovery of an active substance extracted from adrenal glands (adrenaline) cannot be bettered (Dale, 1948; Schäfer, 1908):

> Dr George Oliver, a physician of Harrogate, employed his winter leisure in experiments on his family, using apparatus of his own devising for clinical measurements. In one such experiment he was applying an instrument for measuring the thickness of the radial artery; and, having given his son, who deserves a special memorial, an injection of an extract of the suprarenal gland, prepared from material supplied by the local butcher, Oliver thought that he detected a contraction, or, according to some who have transmitted the story, an expansion of the radial artery. Which ever it was, he went up to London to tell Professor Schäfer what he thought he had observed, and found him engaged in an experiment in which the blood pressure of a dog was being recorded; found him, not unnaturally, incredulous about Oliver's story and very impatient at the interruption. But Oliver was in no hurry, and urged only that a dose of his suprarenal extract should be injected into a vein when Schäfer's own experiment was finished. And so, just to convince Oliver that it was all nonsense, Schäfer gave the injection, and then stood amazed to see the mercury mounting in the arterial manometer till the recording float was lifted almost out of the distal limb.

Within a few months, Oliver and Schäfer had demonstrated that the primary effect of the extract is a profound arteriolar constriction with a resulting increase in the peripheral resistance (Oliver and Schäfer, 1894, 1895). Their colleague Benjamin Moore reported that the activity could be transferred by dialysis through membranes of parchment paper, that it is

Schäfer's own account of his first meeting with George Oliver relates events that may have taken place on an another planet from Dale's version (Schäfer, 1908):

> In the autumn of 1893 there called upon me in my laboratory in University College a gentleman who was personally unknown to me, but with whom I had a common bond of interest – seeing that we had both been pupils of Sharpey, whose chair at that time I had the honour to occupy. I found that my visitor was Dr George Oliver, already distinguished not only as a specialist in his particular branch of medical practice, but also for his clinical applications of physiological methods. Dr Oliver was desirous of discussing with me the results which he had been obtaining from the exhibition by mouth of extracts of certain tissues, and the effects which these had in his hands produced upon the blood vessels of man, as investigated by two instruments which he had devised – one of them, the haemodynamometer, intended to read variations in blood pressure, and the other, the arteriometer, for measuring with exactness the lumen of the radial or any other superficial artery. Dr Oliver had ascertained, or believed that he had ascertained, by the use of these instruments, that glycerin extracts of some organs produce diminution in calibre of the arteries, and increase of pulse tension, of others the reverse effect.

insoluble in organic solvents but readily soluble in water, resistant to acids and to boiling, etc. (Moore, 1895).

HORMONES AND NEUROTRANSMITTERS

> These chemical messengers…or "hormones" (from ὁρμαω, meaning I excite or I arouse), as we may call them, have to be carried from the organ where they are produced to the organ which they affect, by means of the bloodstream, and the continually recurring physiological needs of the organism must determine their repeated production and circulation throughout the body. ***Ernest Henry Starling (1905)***

Hormones are blood-borne "first messengers," usually secreted by one organ (or group of cells) in response to an environmental demand to signal a specific response from another. The first such messenger to be endowed with the title of hormone was secretin, later shown to be a peptide released into the bloodstream from cells in the stomach lining, indicating the presence of food and alerting the pancreas. In the words of William Maddock Bayliss (codiscoverer with Ernest Henry Starling),

> There are a large number of substances, acting powerfully in minute amount, which are of great importance in physiological processes. One class of these consists of the hormones which are produced in a particular organ, pass into the blood current and produce effects in distant organs. They provide, therefore, for a chemical co-ordination of the activities of the organism, working side by side with that through the nervous system. The internal secretions, formed by ductless glands, as well as by other tissues, belong to the class of hormones. ***Bayliss (1924)***

In fact, adrenaline, the signal for fright-fight-or-flight, is a much better candidate for the accolade of "first hormone" (Cannon, 1929). Together with noradrenaline it is secreted into the bloodstream in consequence of emotional shock, physical exercise, cold, or when the blood sugar concentration falls below the point tolerated by nerve cells. Extracts having the activity of adrenaline, enhancing the force and volume of the heart output had been reported 10 years before the discovery of secretin, almost

ADRENALINE VERSUS EPINEPHRINE

It has been customary in Europe to give the substance 4-[1-hydroxy-2-(methylamino)ethyl]-1,2-benzenediol, alternatively 3,4-dihydroxy-α-[(methylamino)methyl]benzyl alcohol the name adrenaline. In the USA, the same substance is called epinephrine. Why have the Europeans employed the Latin (ad ren(es)) roots while the Americans go for the Greek (epi nephr(os))? John Jacob Abel, America's first professor of Pharmacology, is credited with the isolation of the first hormone from adrenal glands as a pure crystalline compound but he failed to determine its composition correctly.

continued

ADRENALINE VERSUS EPINEPHRINE *(cont'd)*

This is credited to Jokichi Takamine, a chemist who had an arrangement with the Parke, Davis Company (Detroit, Michigan), who provided the formula $C_9H_{13}NO_3$. The company lost little time in protecting (by a patent) and marketing the preparation under the trademark of "adrenalin" (no terminal "e"), thereby, preventing the use of the same name by the American scientific community. Abel gave the alternative name *epinephrin* (again, no terminal "e") to his compound. Independent reports from Dakin and Stolz of complete chemical syntheses of the racemic mixture of the two optically active isomers came in 1904 and 1905 (Stolz, 1904; Dakin, 1905). The European Pharmacopoeia now also indicates the use of the term epinephrine.

We justify our preference for adrenaline not only on its historical primacy but also for reasons of logic and common usage. Who ever heard of epinephric receptors? Who ever used the expression "that really gets my epinephrine up"?

simultaneously, by George Oliver and Edward Schäfer in London (Oliver and Schäfer, 1894, 1895), and by Napoleon Cybulski and Szymonowicz in Krakow (Bilski and Kaulbersz, 1987).

In comparison with the ready acceptance of the principle of blood-borne transmission of chemical signals between organs, the idea of chemical (neurotransmitter-operated), as opposed to electrical transmission of impulses between nerves and nerves, and between nerves and muscles had a long gestation. The phenomenon of vagal stimulation causing a slowing of the heart, had been described in 1845 by Weber (see Bacq's account in 1975) and the possibility of chemical transmission of this signal was proposed as early as 1877 by Dubois-Reymond:

> Of known natural processes that might pass on excitation, only two are, in my opinion, worth talking about: either there exists at the boundary of the contractile substance a stimulatory secretion in the form of a thin layer of ammonia, lactic acid, or some other powerful stimulatory substance; or the phenomenon is electrical in nature.

It took all of 60 years for the principle of chemical transmission to achieve acceptance as the general means of communication between nerves and muscles. Otto Loewi recorded (Loewi, 1960) that he had conceived the idea of chemical transmission between nerves as early as 1903, but that at that time (Davenport, 1991).

> I did not see a way to prove the correctness of this hunch, and it entirely slipped my memory until it emerged again in 1920… The night before Easter Sunday of 1920,

> I awoke, turned on the light, and jotted down a few notes on a tiny slip of paper. Then I fell asleep again. It occurred to me at six o'clock in the morning that during the night I had written down something most important, but I was unable to decipher the scrawl. The next night, at three o'clock, the idea returned. It was the design for an experiment to determine whether or not the hypothesis of a chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart according to the nocturnal design.

Henry Hallett Dale and **Otto Loewi** shared the Nobel Prize in 1936 for "their discoveries relating to chemical transmission of nerve impulses."

Henry Dale also contributed to the discovery of oxytocic action of pituitary extracts and he greatly contributed to the understanding of histamine-induced anaphylaxis and on the general conditions of shock.

Loewi relates how, in his experiment, he induced contractions by electrical stimulation of the vagal nerve in an isolated heart. He then transferred some of the fluid from this heart into the ventricle of a second heart undergoing similar stimulation. The result was to slow it down and reduce the force of contraction. He gave the name "vagusstoff" to the inhibitory substance, later identified as acetylcholine.

One might have thought that this demonstration of chemical, not electrical communication between hearts should have settled the issue. However, it is doubtful whether the technique that he used could have delivered the results that he described. There were problems of reproducibility, which may relate both to the temperature of the laboratory and to the seasonal variations in the response of the amphibian heart. In the winter months, the inhibitory fibers predominate so that electrical stimulation suppresses the rate and force of contraction. In the summer, the situation is reversed. Other problems arise from the transient nature of the pulse of the neurotransmitter. Eventually, with the efforts of many others, these difficulties were overcome. Even so, in order to prove the role of acetylcholine in neurotransmission it remained necessary to demonstrate its presence in the relevant presynaptic nerve endings. Also, it was essential to establish that it is actually released when the nerve is stimulated electrically.

One of the main problems confronting all ideas concerning chemical transmission was the instability of the transmitter substance acetylcholine. This had already been synthesized in the mid-nineteenth century. René de M Taveau, previously associated with J. J. Abel, showed that of 20 derivatives of choline, the acetyl ester is the most active in reducing heart rate and blood pressure, an effect opposed by atropine (Hunt and Taveau, 1906). It was first isolated from natural sources in 1914 by Arthur

Ewins, a member of the laboratory of Henry Dale as a component present in an extract of ergot. At the time of his appointment to a post at the Wellcome Physiological Laboratories in 1904, Dale remarks that his employer requested that

> when I could find an opportunity for it without interfering with plans of my own, it would give him a special satisfaction if I would make an attempt to clear up the problem of ergot, the pharmacy, pharmacology and therapeutics of that drug being then in a state of obvious confusion… I was, frankly, not at all attracted by the prospect of making my first excursion into (pharmacology) on the ergot morass.
> ***Dale (1953)***

ERGOT

Although recognized as the "noxious pustule in the ear of grain" over 2500 years ago, written descriptions of ergot poisoning did not appear until the Middle Ages. Ergot is a fungal parasite, *Claviceps purpurea*, that affects grains, particularly rye. The symptoms of ergot poisoning include hallucinations, burning pains in the limbs followed by gangrene, the tissue becoming dry and being consumed by the "Holy Fire, blackened like charcoal." Gangrene is the consequence of prolonged vasoconstriction of peripheral vasculature. Further complications included abortion and convulsions. It has been suggested (Camporesi, 1989) that the St Vitus dance, best described as rave parties of the Middle Ages, may have been a manifestation of the seasonal starvation that occurred during the early summer months. This was the time of the "hungry gap" when the grain stocks were awaiting replenishment with the August harvests. The disease was especially tragic as it was most likely to strike the poorest individuals and intensified the worst harvests—rye is extremely cold-tolerant and was, for many Europeans, food of last resort. Relief was obtained at the shrine of St Anthony, where, perhaps fortuitously, sufferers received a diet free of contaminated grain. Today, the most well-known components of ergot must be the hallucinogen, lysergic acid diethylamide (LSD), and ergotamine, which is sometimes used in the relief of migraine (because of its vasoconstrictive properties).

For Henry Dale the extracts of ergot presented a veritable cornucopia of active substances, to which he returned repeatedly over several years. It was an impurity in a sample of ergot sent to him in 1913 for routine quality control (probably due to contamination by *Bacillus acetylcholini*: fresh ergot does not contain acetylcholine) (see Bacq, 1975), that led him back to the question of transmission at the contacts between nerves and cells. When injected into the vein of an anesthetized cat, the extract caused profound inhibition of the heart beat and being obviously unsuitable for release as a drug, he obtained the whole batch for further investigation in his laboratory. The first thoughts were that the active constituent might be the stable compound muscarine, but on isolation it was found to be the profoundly labile acetylcholine. In a letter to Elliott he writes:

> We got that thing out of our silly ergot extract. It is acetylcholine and a most interesting substance. It is much more active than muscarine, though so easily hydrolysed that its action, when it is injected in the blood-stream, is remarkably evanescent, so that it can be given over and over again with exactly similar effects, like adrenaline (the subject of Elliott's research, see section about the receptive substance). Here is a good candidate for the role of a hormone related to the rest of the autonomic nervous system… ***Letter, Dale to Elliott, December 11, 1913, Contemporary Medical Archives Centre, Wellcome Institute, GC/42 "T.R. Elliott," quoted in Bennet (2000).***

Eserine or physostigmine, an alkaloid isolated from the Calabar bean, had previously shown by Loewi to be an acetylcholinesterase inhibitor.

John Eccles, awarded the Nobel Prize together with Andrew Huxley and Alan Hodgkin in 1963 for discoveries concerning the ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane.

At that time, and even later when it was identified as a chemical component in nonneural tissues, there was never a hint that acetylcholine might have physiological functions. This was not even suggested by the finding as late as 1930 that arterial injections of acetylcholine could induce contractures in denervated muscles. There were still a number of real problems to be overcome. Chief among these was the transient nature of the pulse of neurotransmitter. Also, it was not sufficient to show that acetylcholine applied from a pipette was capable of inducing a response. To prove its role in neurotransmission, it still remained necessary to demonstrate its presence in the relevant presynaptic nerve endings and then to show that it is released upon electrical stimulation.

Feldberg describes his introduction of eserine and the use of a leech-muscle preparation treated with eserine as a specific and sensitive device for measuring the acetylcholine present in the various effluents (blood, organ perfusate, etc.). This was the key that opened the way to the eventual conversion of that most obdurate, skeptic electrophysiologist John Eccles. Even so, without naming any names, Zenon Bacq (1975) reports that even as late as 1950, certain eminent physiologists were still refusing to incorporate the theory of chemical transmission into their teaching.

Hormone-inspired robots

The wisdom brought to light by the proto-endocrinologists, and later expanded by thousands of endocrinologists, has not only led to a thorough understanding of how organisms function and how errors in cellular communication lead to disease, it also provided a rational approach for the development of a rather clever, hormone-inspired, self-reconfigurable robot. CONRO, for configurable-robot, is developed at the ISI institute of the University of Southern California. It is made of separate but connected modules, each operating in its own way, but at the same time having the capacity to cooperate and perform locomotion and self-reconfiguration tasks (see Figure 1-9 for two different configurations). They achieved this by developing an adaptive communication model that mimics the essential properties of hormone-based signaling (a nice example of biomimetics). According to Wei-Min Shen and Peter Will, from a computational point of view, a hormone signal is similar to a content-based message but has the following unique properties: (1) it has no specific destination, (2) it propagates throughout a network, (3) it may have a lifetime, and (4) it may trigger different actions for different receivers (depending both on

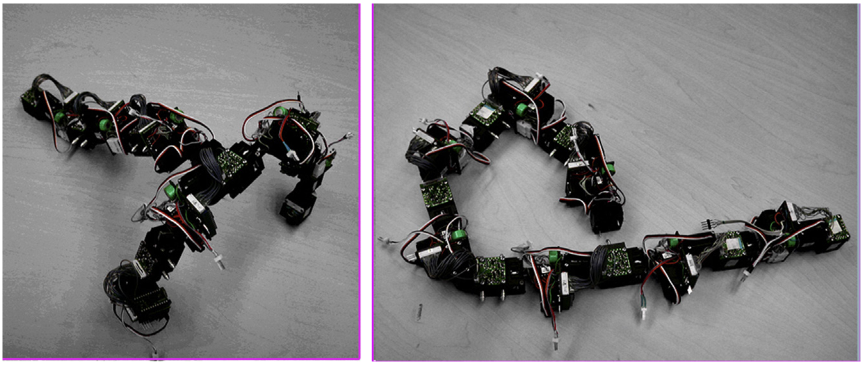


FIGURE 1-9 CONRO, a hormone-inspired self-reconfigurable robot. A modular self-reconfigurable robot has been developed by mimicking the concept of hormone-based signaling. Note the two different configurations: on the left a butterfly-like configuration, allowing rapid movement, on the right a snake-like, allowing passage through very narrow spaces (but at a slow pace).

specific receptor-coupled effector mechanisms and on the local context, like signals from other sensors and the state of the module). Biologists could not have bettered the computer scientists' accurate description of hormone action (Shen et al., 2002, 2004).

For a brief but revealing video of how complex schemes of stimulus-response coupling can lead to rather sophisticated behavior of robots: http://www.isi.edu/robots/movies/MankinCONROWeb.mov.

THE RECEPTIVE SUBSTANCE

As the list of mediators grew, hormones and neurotransmitters, the question followed how they act on sensitive tissues. Important progress in this matter came from two lines of research, one from the field of immunology, the other from physiology. Essential is the notion that cause and effect is the consequence of molecular interaction; the action of medicaments or hormones is a manifestation of their specific "affinity" for particular constituents of cells. In the words of Paul Ehrlich "corpora non agunt nisi fixate," translated as "a component cannot act if it is not bound."

When Ehrlich took on medical education, around 1870, Germany was at its zenith of colonial expansion, and confronted with nasty diseases in far-flung outposts. Moreover, industrial preparation of textile dyes had just become a booming business. Companies like Hoechst, AGFA, and BASF took their origin in dye production, in particular those derived from aniline, a product obtained from distillation of coal tar (creosol). Aniline and methylaniline (toluidine) had become fashionable compounds when, in 1856, William Henry Perkin (an 18-year student of August Hofmann at the Royal College of Chemistry) had produced a purple colorant known under the name of aniline purple. Microscopists too became interested in these dyes as a way to distinguish between cells or cellular organelles. Among these was Ehrlich, a medical student, who wrote his doctorate dissertation on the subject of "Contributions to the Theory and Practice of Histological Staining" (Figure 1-10). One of the most outstanding results of his doctorate investigations was the discovery of the mast cell, rich in heparin-containing granules that bind toluidine blue. Thanks to the application of textile dyes, a subdivision of granulocytes was discovered, those that strongly stained with eosin (eosinophils), those that stained with hematoxylin (basophils), and those that hardly stained at all (neutrophils). At a later stage, Ehrlich also developed an improved staining for the freshly discovered tubercle bacillus (*Mycobacterium tuberculosis*, Robert Koch in 1882) using aniline, fuchsine, and gentian violet (Sakula, 1882).

The word goes that the cell specificity of staining had instigated Ehrlich's idea that textile dyes could possibly be used in chemotherapy. Here we employ the term in its original broad sense: the use of synthetic

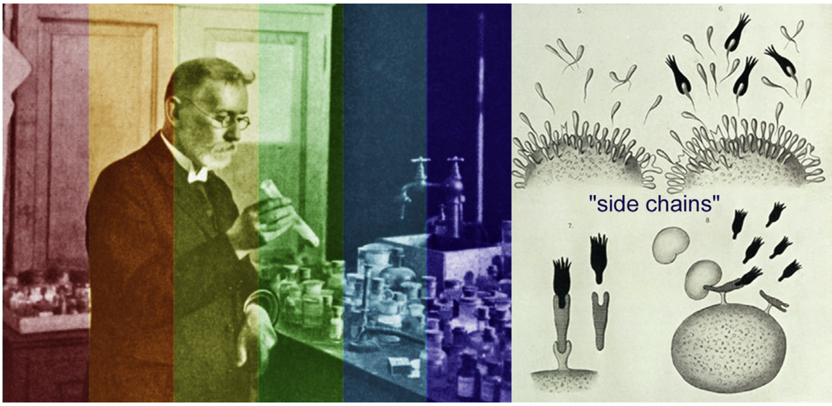


FIGURE 1-10 Textile dyes are the very origin of Ehrlich's theory of side chains (receptive substance). These beautiful diagrams illustrating Paul Ehrlich's side-chain theory featured in his Croonian Lecture "On Immunity with Special Reference to Cell Life," which he gave to the Royal Society on March 22, 1900. His theory was later proved to be wrong, but his images left an indelible mark on researchers, inspiring them to investigate the true nature of these mysterious antibodies. *Image of Ehrlich modified from Waldemar Titzenthaler, 1910, appeared in* Berliner Illustrirte Zeitung *31/1910. Source de KRUIF, P. Mikrobenjäger. Orell Füssli, Zürich, 1927. Image of "side chains" kindly provided by Wellcome Library, London.*

chemicals working together with the body's own defenses to combat infection or new growth (neoplasia). Thus he showed that trypan red has some effect on *Trypanosoma equinum* (but not on the human pathogens *brucei* and *cruzi*). Methylene blue was shown to block gametocyte development of *Plasmodium falciparum* and was introduced as an antimalaria agent (Adjalley et al., 2011). It eventually eclipsed because soldiers fighting in the Pacific battleground of World War II strongly disliked its prominent adverse effects: it turned their eye balls blue and their urine green. Two very successful medicaments are a direct, although being delayed, outgrowth of Ehrlich's postulation, namely prontosil (antibacterial agent obtained from the red dye sulfonamidochrysoidine) and suramin (an antitrypanosomiasis compound derived from the trypan dyes) (Wainwright, 2010). A last example, unrelated to Paul Ehrlich, Paris green, an arsenite-containing dye, had been successfully employed to control the Colorado potato beetle back in 1867.

Perhaps most noticeable is Ehrlichs' contribution, together with Sahachiro Hata, to the discovery of a first treatment against syphilis. In a long and highly systematic search for compounds that cured trypanosome infection (sleeping sickness) in mice, they found an effective organic arsenic compound named arsphenamine (number 606 in a range of over 915 compounds scrutinized). It was Hata's contribution to show that it was much more effective against *Treponema pallidum*. Within 1 year of discovery, it was commercialized by Hoechst under the name of Salvarsan. Indeed, an "arsenic salvation" it must have been for all those suffering from syphilis. The compound rapidly made its fame (or ill fame, depending on how much one suffered from its adverse effects) until superseded by penicillin in the 1940s.

In his Nobel Lecture (1908) Ehrlich joins rank with Thomas Huxley when he proclaims:

> …that for a further penetration into the important, all-governing problem of cell life even the most highly refined optical aids will be of no use to us. Now, at this moment, the time has come to penetrate into the most subtle chemism of cell life and to break down the concept of the cell as a unit into that of a great number of individual specific partial functions. But since what happens in the cell is chiefly of a chemical nature and since the configuration of chemical structures lies beyond the limits of the eye's perception we shall have to find other methods of investigation for this. This approach is not only of great importance for a real understanding of the life processes, but also the basis for a truly rational use of medicinal substances.

PRONTOSIL

Sulfonamidochrysoidine, a red dye, was first synthesized by Bayer chemists Josef Klarer and Fritz Mietzsch as part of a research program designed to find dyes that might act as antibacterial drugs in the body. The molecule was tested and in the late autumn of 1932 was found effective against some important bacterial infections in mice by Gerhard Domagk. It was marketed as prontosil and is now considered being the first effective antibiotic (with more or less acceptable adverse effects). In late 1935, the group of Ernest Fourneau, at the Institut Pasteur in Paris, showed that prontosil becomes active only after metabolic processing in which sulfanilamide is produced. This specifically blocks folate production in bacteria, hence preventing their DNA synthesis (acting as bacteriostatic agent). Sulfanilamide served as a lead for the production of new sulfonamide antibiotics of which sulfamethoxazole is still in use (component of co-trimoxazole).

DYES AND NAMING MICROBES

To illustrate once again how much dyes influenced the scientific enterprise at the end of the nineteenth century, *T. pallidum* was thus named because of its pallid appearance. The bacterium proved to be difficult to stain with textile dyes. Current diagnosis protocols include dark-field microscopy, immunostaining (*Treponema*-specific antibodies), polymerase chain reaction, and, sometimes, a rabbit infectivity test.

continued

DYES AND NAMING MICROBES *(cont'd)*

Trypan blue, derived from *o*-methylaniline (*o*-toluidine), is so-called because of its blue color and its capacity to kill Trypanosomes, the parasites that cause Trypanosomiasis or sleeping sickness. The members of the genus *Trypanosoma* obtained their name because of their corkscrew or drill-bit-like motion (from Greek, *trypanon,* borer, *soma,* body). Trypan blue is a diazo dye, also known as diamine blue and Niagara blue. Trypan red and trypan blue were first synthesized by Paul Ehrlich in 1904. A remote analog of trypan dyes, suramin, is still used in the treatment of sleeping sickness.

Reference; Web site dedicated to Paul Ehrlich http://www.paul-ehrlich.de/.

However, it was during his studies on therapeutic sera (starting around 1890), in particular dealing with a treatment for diphtheria, that Paul Ehrlich elaborated the idea of a "receptive side chain." He reasoned that the sera (which he obtained through collaboration with Emil von Behring) must contain substances that first bind with and subsequently inactivate bacterial toxins. These substances have entered the serum, after an animal or human is exposed to the pathogenic agent. To explain how they came about, Ehrlich proposed that certain membrane proteins, which he named "side chains" of a particular cell have an "atom group" that, by mere coincidence, possesses specific combining properties for a particular toxin. Upon binding, both the side chain and the toxin lose their activity. In order to make up for the loss of activity, the cell starts to produce large amounts of the side chain. Many of the excess side chains can now break off and are released into the blood stream, thus acting as soluble antitoxins; hence the protective action of serum (the soluble component of blood after clotting). He assumed that the antipodes, toxin and receptive side chain, engage in a chemical bond, albeit reversible, through the principle of "lock and key" (a comparison made by Emil Fischer). We must credit Ehrlich not only for his systematic approach to screening of medicaments but also for his excellent sense of imagination. Although his theory does not rhyme with our current understanding of immunity, receptor targets for toxins and neutralizing immunoglobulins (antibodies) are quite distinct entities, Ehrlich's theory of receptive side chains provided a strong impulse for the development of vaccination and passive immunization protocols. Furthermore, it opened the way to search for toxin receptors, the target by which they interact with the infected organism and cause disease.

Quite surprisingly, Ehrlich initially refused to apply the receptive side chain theory to his earlier studies with dyes. He argued that the easy reversibility of staining (removed by alcohol, acetone, or water) made it unlikely that the dyes bound “receptive side chains” (Croonian lecture, 1900). He gradually changed his mind while reading about Langley’s “receptive substance,” which bound even smaller molecules like nicotine. Around 1907, Ehrlich introduced the words “chemoreceptor” and “poison receptor” or just “receptors” (Nobel Lecture, 1908). The passage below, from his Nobel Lecture, witnesses how much Ehrlich’s vocabulary resembled that of the Langley (or how two disciplines, immunology and physiology had converged to point to a unifying concept):

> when we see how some bacterial poisons produce disturbance only after weeks of incubation and then damage the heart or the kidney or nerves, when we see how animals suffering from tetanus present contractions and spasms for months, we are forced to the direct conclusion that all these phenomena can only be caused by the adhesion of toxic substance to quite definite cell complexes. I therefore assumed that the tetanus toxin for instance, must unite with certain chemical groupings in the protoplasm of cells, particularly the motor ganglion cells, and that this chemical union represents the prerequisite and cause of the disease. I have therefore simply called such groupings “poison receptors” or just “receptors.”

Ehrlich shared the Nobel Prize in Physiology or Medicine in 1908 with Ilya Mechnikov “in recognition of their work on immunity.”

Gerhard Domagk received the Nobel Prize in Physiology or Medicine in 1939 “for the discovery of the antibacterial effects of prontosil.”

Emil von Behring received the Nobel Prize in Physiology or Medicine in 1901 “for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths.”

John Newport Langley, who remained throughout his whole career at the University of Cambridge, took over where Claude Bernard, professor in medicine at “College de France” in Paris, had left the stage, namely, to sort out where exactly did curare and nicotine (or atropine and pilocarpine) act in the stimulus-response coupling between nerves and striated (or smooth) muscle (Figure 1-11)? Bernard had made the crucial observation that when frog muscles are paralyzed by curare, they fail to respond to electric stimulation of the motor nerve but they still contract when the current is applied directly to muscle. In order words, the contractile machinery of actin and myosin is not affected by curare (Maehle et al., 2002; Bennet, 2000).

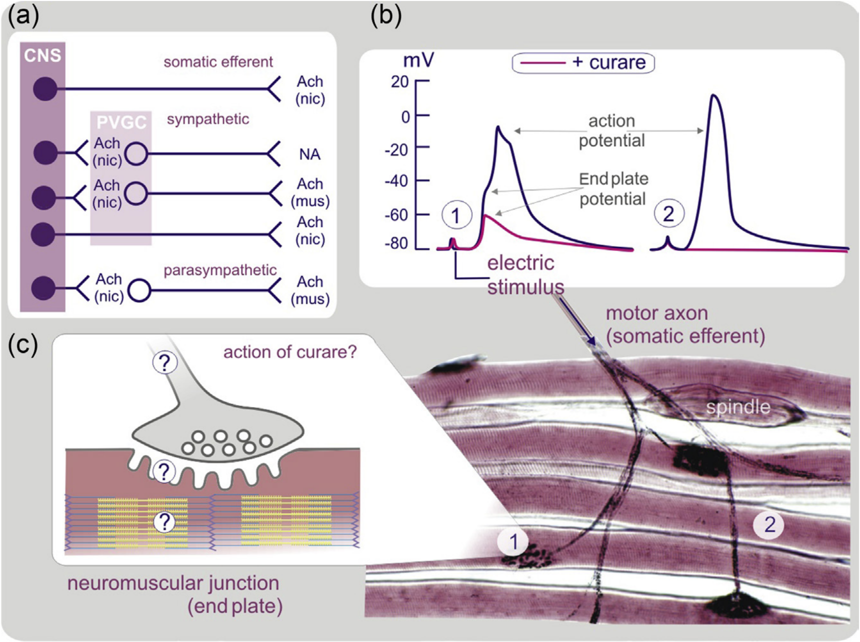


FIGURE 1-11 Langley's mission: Where does tubocurarine affect the neuromuscular transmission? (a) Acetylcholine and noradrenaline as transmitters in the peripheral nervous system. CNS, central nervous system; PVGC, paravertebral ganglion chain; Ach, acetylcholine; nic, nicotinic; mus, muscarinic; NA, noradrenaline. (b) Microelectrode recording at the end plate (1) and away from the end plate (2) in the muscle fiber. Tubocurarine reduces the end plate potential so that no action potential is generated (muscle paralyzed). (c) Langley had to sort out where exactly tubocurarine acted. We now know that tubocurarine blocks the binding of acetylcholine to its receptor (the nicotinic acetylcholine receptor located in the postsynaptic membrane (sarcolemma)). *Image of muscle fibers from Dr T Caceci (Virginia–Maryland College of Veterinary Medicine).*

He approached the question in different experimental settings over a period of 30 years, with an interlude of 20 years during which he investigated the secretion of pepsinogen and pepsin. Concerning stimulus-response coupling, following an assignment by his supervisor Professor Michael Forster, Langley commenced his career with the elucidation of the effects of the drug jaborandi (pilocarpine) in frog hearts and submaxillary glands from cats and dogs. This short prelude already made him conclude that pharmacological agents form compounds (*chemical groupings* in the words of Ehrlich) with a substance in cells. In his article in 1878 he comments (Langley, 1878):

> we may, I think, without much rashness, assume that there is a substance or substances in the nerve endings or gland cells with which both atropine and pilocarpine are capable of forming compounds. On this assumption then the atropine or pilocarpine compounds are formed according to some law of which their relative mass and chemical affinity for the substance are factors.

If we replace "mass" with "concentration," the last sentence can serve as well today (providing the essential parameters for the Schild equation) as when it was written (Jenkinson, 2011). For the good order, atropine and pilocarpine are, respectively, an antagonist and agonist of the muscarinic acetylcholine receptor: atropine causes drying of the mouth by blocking the parasympathetic-mediated secretion (cholinergic nerves) and it increases heart rate by blocking the inhibitory influence of the vagus nerve. Pilocarpine, in contrast, evokes copious flow of saliva and increases the heart rate.

ORIGIN OF THE TERM (ORTHO) SYMPATHETIC AND PARASYMPATHETIC

It was Jacob Benignus Winslow (1669–1760), Danish-born Parisian anatomist, who in 1716 renamed the "intercostal nerve" (now called paravertebral ganglion chain) to "great sympathetic nerve." In fact, because of its numerous communications with most of the major nerves of the whole body, this structure was considered by Winslow as being "sympathetic" with other neural structures. Subsequently, Langley introduced the term "parasympathetic innervations" in order to distinguish the cranial nerves (among which the vagus nerve) from the sympathetic ones. Sympathy, both neural and humoral, is an old notion, it accounts for the cooperation or coordination of organs.

THE SOMATIC AND AUTONOMIC NERVOUS SYSTEM

For a concise overview of the somatic and autonomic nervous system, its chemical mediators and the numerous medicaments that affect their function, we suggest Chapters 9–11 of "Pharmacology" (Rang et al., 2007).

CURARE

Curare is mixture of naturally occurring alkaloids found in various South American plants and used as arrow poisons. The most important component is tubocurarine, the structure of which was elucidated in 1935 (purified from *Strychnos toxifera*). Tubocurarine qualifies as nondepolarizing blocking agent: it acts as a competitive antagonist of the nicotinic Ach receptor in the postsynaptic membrane of the end plate.

Roughly 20 years later, Langley returns to the subject of nervous transmission this time in the context of the efferent somatic system (motor neurons) and with the results of Elliott firmly in the back of his mind. Thomas R. Elliott, a student from Langley, had shown that adrenaline (or suprarenal extracts) led to contraction of smooth muscle (intestinal tract) in a similar fashion as did stimulation of the sympathetic nerves. The action persists even when the sympathetic neurons were degraded and he concluded that "adrenaline might then be the chemical stimulant liberated on each occasion when the (nerve) impulse arrives at the periphery" (Elliott, 1905). In short, Elliott proposed that adrenaline, which qualified as a hormone, could also act as a neurotransmitter. We now know that this is noradrenaline (see Figure 1-11(a)).

Langley applied similar experiments to anesthetized fowl and measured contraction of the gastrocnemius muscle following injection of nicotine into the bloodstream. In order to remove nerve innervations, the same experiments were repeated with animals from which the sciatic and crural nerves were cut weeks in advance (leading to full degeneration). Upon injection of nicotine, the animals stretch their legs (and open their eyes) and this is undone by the subsequent injection of curare (Figure 1-12). However, direct electric stimulation of the same muscle still leads to contraction. "It follows then that curare acts upon the muscle substance and not upon the axon-endings. Since, in the normal state, both nicotine and curare abolish the effect of nerve stimulation, but do not prevent contraction from being obtained by direct stimulation of the muscle or by further adequate injection of nicotine, it may be inferred that neither the poisons nor the nervous impulse act directly on the contractile substance of the muscle but on some accessory substance. Since this accessory substance is the recipient of stimuli which it transfers to the contractile material, we may speak of it as the receptive substance of the muscle" (Bennet, 2000; Langley, 1905).

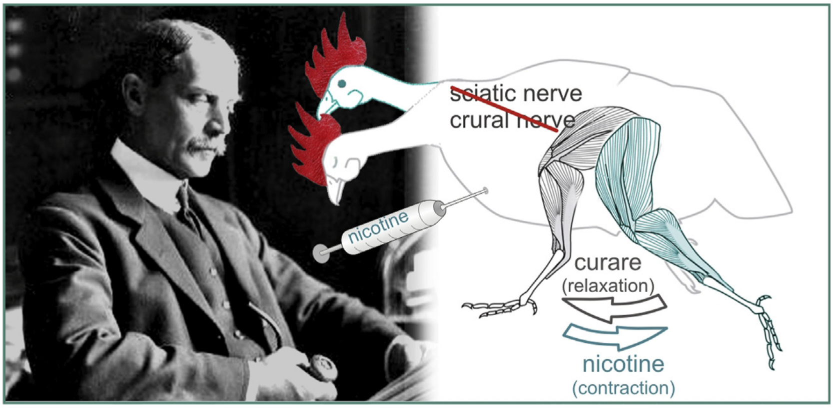


FIGURE 1-12 Langley and the experimental setup that led him to the enunciation of the receptive substance.

Apparently, Langley disliked speculating and developing hypothesis and he has left no graphical representations of his (private) mental pictures. Had he be forced to draw his receptive substance in relation to the contractile apparatus, in the way Ehrlich has done for the relationship between toxin and the side chain (Figure 1-10), we would have learned that the proposed mechanism was far from correct. Langley only gives a hint about a possible mode of action in 1908 by stating:

> My theory of the action is in general in lines of Ehrlich's theory of immunity. I take it that the contractile molecule has a number of "receptive" or "side-chain" radicles and that nicotine, by combining with one of these, causes contraction and, by combining with another, causes twitching… ***Bennet (2000)***

The term "receptor" does not yet appear in the index of the 1955 edition of *The Pharmacological Basis of Therapeutics*, the standard American textbook of pharmacology, but the following sentence was there: "Years ago, Langley named the differentiating substance the 'receptive substance'; this term is still widely employed, but it must be realized that the 'receptor' may not be a morphologically demonstrable structure" (Goodman and Gilman, 1955; Nobel speech AG Gilman, 1994). Perhaps, that is why Langley was often nominated but never rewarded a Nobel Prize for his discoveries and so we pay our tribute to his pioneering work with a fragment from his obituary:

> Each gain he made was a step placed securely and finally and few indeed of them, as the road has become form firmly and widely trodden by others following, have been found wrongly placed. All his chief words keep, and must always keep their place in the significant history of Animal Physiology. The bare titles of his papers and books deployed there along the years give the plainest testimony to me to his unvarying, unhalting service to science. No single year in all that series, extending will nigh for half a century, from youth to age, appears without its contribution of effective work. ***Fletcher (1926)***

More importantly of course is that Langley's concept of the receptive substance was taken up by others, notably Dale and Loewi with respect to chemical synaptic transmission. By 1970, the nicotinic acetylcholine receptor was purified from the electric organ of Torpedo (Miledi et al., 1971; Changeux et al., 1970). In that year Katz and Miledi, using micropipettes, demonstrated that addition of acetylcholine was linked to changes in membrane conductance in single synapses (Katz and Miledi, 1970). It took longer for the adrenergic receptor, as there are no tissues that express it so abundantly as the nicotinic acetylcholine receptor in Torpedo, but by 1984 it was shown that reconstitution of the pure receptors with G-proteins and adenylyl cyclase was sufficient to create a neurotransmitter responsive system (Cerione et al., 1984) and by 1986 the first mammalian beta-adrenergic receptor was cloned (Dixon et al., 1986).

PROTO-MESSENGERS AND -RECEPTORS

It is obvious that the very first stimulus to a presumed primordial organism must have been from the immediate environment, signals that hinted to the presence or absence of conditions, which allowed or prevented replication. Bacteria have developed sophisticated chemotactic responses to nutrients, connecting membrane receptors with flagellar motors (Bren and Eisenbach, 2000). Molecules that we now regard as messengers (extra- or intracellular) of our bodies are of great antiquity on the biological timescale. It is interesting to consider which came first: the messengers or the receptors that they control? The general view is that the messengers, and their pathways for biosynthesis, preceded the development of concomitant receptors as we know them in mammals. An explanation for this order of appearance may be explained by an as yet hypothesis that receptors occurred as a consequence of convergence of fragments of genes involved in metabolic pathways. For instance, it has been shown that the human folate receptor-α (FOLR1), a membrane transport protein, carries sequence homology with several enzymes involved in folate synthesis in *Saccharomyces cerevisiae* (among others: polyglutamate synthase, folic acid synthesis protein FOL-1, dihydrofolate reductase, GTP cyclohydrolase 1, and folylpolyglutamate synthase) (Ramamoorthy and Shanker Verma, 2008). There is a sense of logic in that, like receptors, metabolic enzymes too will have to recognize the metabolites and one may assume that the number of binding pockets (suitable biochemical space) that nicely fits folate (or an intermediate product) is limited. These newly formed binding sites then connect with signaling or transporter activities and new functions appear that are entirely unrelated to the metabolic pathways (although they may affect them in one way or another).

Folate, from Latin folium, is present in the leafs of fresh vegetables. Folate (folic acid or water-soluble vitamin B9) is an essential component for nucleotide synthesis (AMP, GMP, TMP) and amino acid metabolism (methionine, histidine, serine). Humans have lost the capacity to synthesize folate and rely on external sources. Deficiency during embryonic development is associated with neural tube defects (spina bifida and anencephaly) (OMIM 601634).

Substances recognized as having the actions of hormones in animals first made their appearance at early stages of evolution. For instance, chemical structures closely related to thyroid hormones have been discovered in algae, in sponges, and in many invertebrates. Steroids such as estradiol are present in microorganisms and also in ferns and conifers. Catecholamines (dopamine and (nor)adrenaline) have been found in protozoa (Janakidevi et al., 1966a,b) and ephedrine, resembling both adrenaline and amphetamine, can be isolated from the stems and leaves of the Chinese herb ma huang (*Ephedra sinica*). What it does to the herb is not described but in humans it causes vasoconstriction (peripheral effect, stimulating the action of adrenaline) and because it readily crosses the blood–brain barrier, it qualifies as an illegal psychostimulant substance (acting as an amphetamine, liberating dopamine and noradrenaline in certain neural synapses). There are claims, based on immunological detection, for the presence of peptides related to insulin and the endorphins in protozoa and fungi (LeRoith et al., 1980) although no signal function has been discerned.

The a- and α-type mating factors of yeast (produced by a- and α-cells, respectively), which certainly act as messengers, are very similar in structure to gonadotropin-releasing hormone that controls the release of gonadotropins from the anterior pituitary in mammals (LeRoith et al., 1983; Hunt and Dayhoff, 1979). Factors resembling the mammalian atrial natriuretic peptide are present in the cytosol of the single cell eukaryote *Paramecium multimicronucleatum* (Vesely and Giordano, 1992) and in the leaves of many species of plants where they act as regulators of solvent and solute flow and of the rate of transpiration (Vesely et al., 1993). ACTH and β-endorphin are present in protozoa. These organisms also contain high MW precursors of these peptides reminiscent of the vertebrate proopiomelanocortin (LeRoith et al., 1981, 1982).

Receptor-like proteins in nonanimal cells have been much harder to identify. A recently described example is a protein expressed in the plant *Arabidopsis* that shares extensive sequence homology with the ionotropic glutamate receptor of mammalian brains (Lam et al., 1998). A corollary arising from this is the possibility that the potent neurotoxins thought to be generated in defense against herbivores may have their origin as specific agonists and were only later selected and adapted in some species as poisons. $GABA_B$-like receptors and Frizzled-like receptors (for the messenger Wnt), which were thought to be exclusive for the animal kingdom, have been detected in slime molds (*Dictyostelium discoideum*). The corresponding messengers remain elusive on this occasion (Prabhu and Eichinger, 2006). Molecules having a close relationship to the receptors for epidermal growth factor (EGF) and insulin apparently evolved in sponges prior to the Cambrian Explosion (more than 600 million years ago) and it has been proposed that they may have contributed to the rapid development of the complex multicellular phyla (Skorokhod et al., 1999).

Amphioxus lanceolatus also called the lancelet is a member of the subphylum *Cephalochordata.* It is a fish-like creature 2 or 3 inches long, found in the shallow depths of temperate seas. Its body is pointed at both ends and it can swim both forward and backward. It is the lowest and most generalized of the vertebrates, having neither brain, skull, nor vertebrae.

These animals are primarily sedentary, although they are able to swim short distances. For most of its life, the amphioxus is buried in the sand, its oral appendages protruding into the water.

Amphioxi are useful in filtering the water and in areas where they abound the water is usually clean.

Although invertebrates express some members of the nuclear receptor family (such as the receptor for thyroid hormone and vitamin D), nuclear receptors for adrenal and sex steroid hormones (cortisol, aldosterone, testosterone, estradiol, progesterone, etc.) are absent (Escriva et al., 2003). The ancestral steroid hormone receptor probably made its first appearance in a cephalochordate such as amphioxus. Receptors for estradiol, progesterone, and cortisol have been cloned from lamprey (fish-like, considered an ancient lineage of vertebrates). From hereon in evolution the steroid hormones would have allowed for a ligand-based mechanism for the regulation of gene transcription and this could have promoted the complex processes of differentiation and development found in the higher vertebrates (Baker, 2003). Thus, the *hox* genes that are critical for development and differentiation, including the brain of amphioxus (Manzanares et al., 2003) are regulated by estrogens and progestins.

An important consequence of the separate arrival of messenger and receptors in the course of evolution is that the responses to a given messenger (be it a hormone or other) can vary widely across different species and even within species. Two examples: numerous actions of prolactin have been identified (Nicoll, 1982). It is the regulator of mammary growth and differentiation and of milk protein synthesis in mammals. In birds, it acts as a stimulus to crop milk production and in some species as a controlling factor for fat deposition and as a determinant of migratory behavior (Meier et al., 1965; Meier, 1977). It is a regulator of water balance in urodeles (newts and salamanders) and of salt adaptation and melanogenesis in fish (Nicoll, 1982). Serotonin (5-hydroxytryptamine), a neurotransmitter that controls mood in humans is reported to stimulate spawning in mollusks, probably as a consequence of its conversion to melatonin (naturally, one wonders whether it affects their mood as well). These examples highlight an important takeaway message, signaling processes have not been designed to serve certain functions, acting in defined stimulus-response circuits; they arose by chance, provided new opportunities (new ecological niches, increased survival, or others) and therefore stood the test of time. Pathways may thus have appeared independently in different species, they may have been transmitted between species through gene transfer mechanisms or they were inherited from ancestors. Certain receptors or signaling components may have disappeared in one species but remained opportune in others. An evolutionary reconstruction of signaling pathways may reveal how they came about, and disappeared, but this lies beyond the scope of this book (see, for instance, Anantharaman et al. (2007) and Schaap (2011)).

GROWTH FACTORS: SETTING THE FRAMEWORK

The trails that led to the discovery of the growth factors (and related messengers) are very different from those that revealed the first hormones and neurotransmitters. For a start, people knew, more or less, what to look for and where to go looking, and in general, the tale is somewhat less romantic and less fraught with angst and vehement disagreements. However, what began with a simple search for factors that would sustain living cells in laboratory conditions has expanded into a plethora of subject areas, which are subjects in their own right. These include inflammation, wound healing, immune surveillance, development, and carcinogenesis. To confront this bewildering prospect, we first set out some details of how it evolved initially so that the reader is aware of how the major questions developed and have been confronted. A difficulty arises from the convergence of different disciplines, each bringing with it the baggage of its favored nomenclature. We return to this matter at the end of this chapter.

Viruses and tumors

The first report of a tumor linked to a virus appeared in 1908, when Ellermann and Bang obtained a filterable agent from a chicken leukemia and were able to make six passages of it, from fowl to fowl, producing the same disease each time (Ellermann and Bang, 1908). Their report was generally disregarded. Leukemia was not considered to be a tumor, though as should have been evident from the work of Aldred Warthin (Figure 1-13), first reporting in 1904 (Warthin, 1904, 1907), a link between leukemias and tumors, and hence cell growth and proliferation, had already been established:

"These conditions are comparable to malignant tumors. The formation of metastases, the infiltrative and destructive growth, the failure of innoculations and transplantations etc., all favor the view that they are neoplasms, and present the same problems as do the malignant tumors."

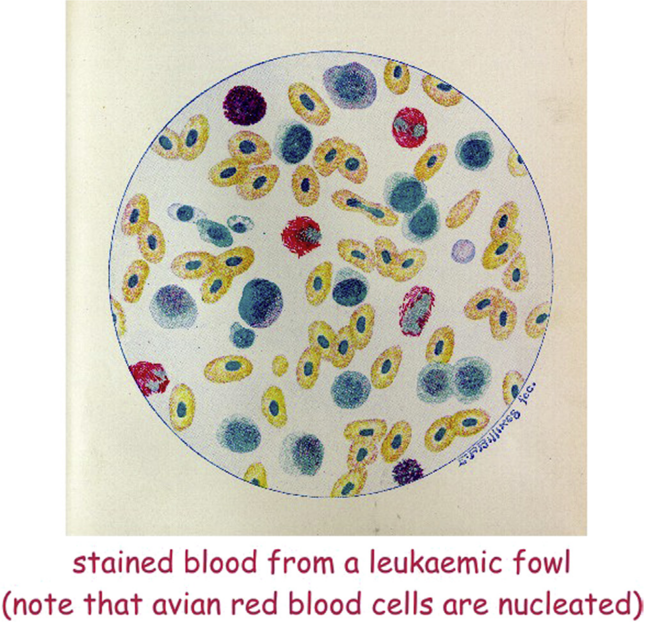

stained blood from a leukaemic fowl
(note that avian red blood cells are nucleated)

FIGURE 1-13 Stained blood smear from a leukemic fowl. “In December 1905, there came into my hands a Buff Cochin Bantam hen showing signs of illness in the way of indisposition to move about and a general weakness of a progressive character. No symptoms of ordinary fowl diseases were present… Examination of blood smears showed, however, a great increase of white cells of the large lymphocyte type… A diagnosis of leukemia was therefore made… A great variety of staining methods were used, including the most recent methods for the staining of spirochetes and protozoan parasites… No evidence of the existence of any infective agent could be obtained” (Warthin, 1907). (Note that avian red blood cells (yellow stain) are nucleated.)

More than 20 years were to pass before the leukemias were eventually recognized as being tumors or neoplastic diseases.

Francis Peyton Rous (1910) described a chicken tumor, identified as a sarcoma, that could be propagated by transplanting its cells, these then multiplying in their new hosts giving rise to tumors of the same sort. The cells yielded a virus, now known as Rous sarcoma virus, from which, in 1980, the first protein tyrosine kinase, v-Src, was isolated (Erikson et al., 1980; Hunter and Sefton, 1980). Writing at the end of his career in 1967, Rous gives an apt description of how things were done:

> Those were primitive times in the raising of chickens. They were sold in a New York market not far from the institute, and many individual breeders brought their stock there. Every week F. S. Jones, a gifted veterinarian attached to my laboratory, went to it, not only to buy living chickens with lumps which might be tumors, but any that seemed sickly and had a pale comb, as perhaps having leukemia. Thus within less than four years we got more than 60 spontaneous tumors of various sorts…

Francis Peyton Rous (1879–1970) shared the 1966 Nobel Prize for Physiology or Medicine for his discovery of tumor-inducing viruses.

Sarcoma is a form of cancer that arises in the supportive tissues such as bone, cartilage, fat, or muscle. A **carcinoma** is defined as a malignant new growth that arises from epithelium, found in skin or, more commonly, the lining of body organs, for example: breast, prostate, lung, stomach, or bowel. Carcinomas tend to infiltrate into adjacent tissue and spread (metastasize) to distant organs, for example: to bone, liver, lung, or the brain.

The discovery of nerve growth factor…and EGF

> Among the fractions that I assayed in vitro the following day, there was one containing snake venom. Having not been told which of the fractions had been specially treated, I was completely stunned by the stupendous halo radiating from the ganglia. I called Stan in without telling him what I had seen. He looked through the microscope's eyepieces, lifted his head, cleaned his glasses which had fogged up, and looked again. "Rita," he murmured, "I'm afraid we've just used up all the good luck we're entitled to. From now on, we can only count on ourselves…" Events were to prove him wrong.

v indicates a viral origin, commonly a transforming mutant. **c** indicates the cellular wild type. **Src**, sarcoma.

Nerve growth factor (NGF) may perhaps be regarded as the first identified growth factor, but there were many early clues hinting at their existence. The embryologist Hans Spemann (Nobel Prize, 1935) had described the eponymous "organizer" that directs the creation of the dorsal axis (development of neural tube, notochord, vertebral discs) in the gastrula stage in the development of the amphibian embryo (see Chapter 17). This work had famously indicated that soluble factors made by the embryonic cells must be instrumental in regulating cell proliferation and differentiation (Hamburger, 1969).

The quotations in this section are taken from *In praise of Imperfection, My Life and Work* by Rita Levi-Montalcini (1988). A facsimile collection of her major papers with annotations by the author is presented in Levi-Montalcini (1997).

Rita Levi-Montalcini's affair with growth factors had its origin at that time. Dismissed from her university post by the Nazi racial edicts, she determined to continue on alone. She had been alerted to the work of Spemann by an article written by one of his protégés, Viktor Hamburger. He described the concept of the inductive reaction of certain tissues on others during early development. In particular, he cited the effect of ablating the embryo limb buds of chicks upon the reduction in the volume of the motor column and the spinal ganglia responsible for the innervation of the limbs. The idea was that the failure of the cells to differentiate and to develop is due to the absence of an inductive factor normally released by the innervated tissues. She aimed to understand how the excision of noninnervated tissue could affect differentiation and subsequent development.

Stanley Cohen and Rita Levi-Montalcini were awarded the Nobel Prize in 1986 "for their discoveries of growth factors."

Confined to working in her bedroom she made use of just the most basic materials and instruments needed for histological investigation. In her examination of this problem, Levi-Montalcini found that nerve cell differentiation proceeds quite normally in the embryos with excised limbs, but that a degenerative process (what we would now call apoptosis) commences as soon as the cells emerge from the cord and ganglia appear at the stump of the amputated limb (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini, 1950). It appeared to her that the failure to develop was best explained by the absence of a trophic factor.

In 1946, she sailed for the USA in the company of Renato Dulbecco (see below), a friend from her student days. She was destined for St Louis, he for Bloomington. Intending to pay a brief visit of a few weeks to the laboratory of Viktor Hamburger, she remained there for 30 years. The work that led eventually to the discovery of NGF had its origins in the observation by a late student of Hamburger's, Elmer Bueker. He had reported that fragments of an actively growing mouse tumor, grafted on to chick embryos, caused a great ramification of nerve fibers into the mass of tumor cells (Bueker, 1948). Seeking advice and encouragement, he suggested that the tumor generated conditions favorable for the differentiation of the nerve cells, which was reflected in the increased volume of the ganglia. Repeating the experiment, Levi-Montalcini describes the extraordinary spectacle of seeing bundles of nerve fibers passing between the tumor cells like rivulets of water flowing steadily over a bed of stones. In no case did they make any connection with the cells, as is the rule when fibers innervate normal embryonic or adult tissue. Later she describes how the sympathetic fibers invaded the embryonal viscera, even entering into lumen of the venous, but not the arterial blood vessels so that the smaller veins were quite obstructed.

> The penetration of the nerve fibers into the veins, furthermore, suggested to me that this still unknown humoral substance might be exerting a neurotropic effect, or what is known as a chemotactic directing force, one that causes nerve fibers to grow in a particular direction… Among these, I guessed, was undoubtedly also the humoral growth factor that the cells produced. This hypothesis would explain this most atypical finding of sympathetic fibers gaining access inside the veins… Now, with the hindsight of the nearly forty years gone by since those moments of keenest excitement – it appears that the new field of research that was opening up before my eyes was, in reality, much vaster than I could possibly have imagined.

It was clearly necessary to develop an *in vitro* assay. These were early days in the field of cell culture and it took the best part of 6 months to develop a practical method that could be used as the basis for measuring the biological activity of fractions in protein purification. Only then was the point reached when a biochemical approach could usefully be applied in the pursuit of NGF, the name by which the factor became known shortly after.

> "Rita," Stan said one day, "you and I are good, but together, we are wonderful."

After a year's intense work, they had narrowed down the factor as a nucleoprotein, though Stanley Cohen suspected that the nucleic acid component was likely to be a contaminant. On the advice of Arthur Kornberg, he applied an extract of snake venom as a source of nuclease activity with the aim of removing nucleic acids, present as an impurity in their material. To their great surprise, this yielded a preparation that enhanced neuronal growth still further. It emerged that the snake venom alone was active (Cohen and Levi-Montalcini, 1958). Making the connection between venom and saliva, they tested mouse salivary glands and found that this too is an excellent source of NGF activity. (If they had been able to purchase purified nuclease enzyme, the course of the discovery must surely have been prolonged.)

Later, Cohen discovered a new phenomenon that "was destined to become a magic wand that opened a whole new horizon to biological studies." A contaminating factor was present that caused precocious

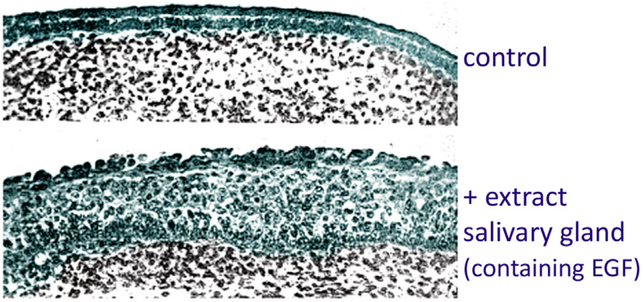


FIGURE 1-14 An early demonstration of the effects of epidermal growth factor (EGF). Thickening of the epidermal layer in skin explants from chick embryos after 3 days in culture (Cohen, 1964).

growth in epidermis (Figure 1-14). It was also found that the factor has a powerful proliferative effect on connective tissues and it became clear that there is a link between the mechanisms that control normal proliferation and neoplastic growth.

Since, under culture conditions, the stimulus to proliferate could not involve systemic or hormonal influences, Cohen called the new protein EGF (Cohen, 1964, 1986). Later, it was shown that mouse EGF enhances DNA synthesis in cultured human fibroblasts. It was also found that EGF is similar to urogastrone, a peptide that had been isolated from human urine and recognized by pharmacologists because of its ability to inhibit gastric acid secretion (Gregory, 1975). Out of 53 residues in the amino acid sequence, 37 share a common location and the 2 polypeptides have similar effects on both gastric acid secretion and the growth of epidermal cells.

All this now paved the way for a more molecular approach using isolated cells. It was found that rat kidney cells, transformed with the Kirsten sarcoma virus fail to bind EGF. This downregulation, which is due to internalization of the receptor, is caused by the elevated expression and release of EGF by the cells themselves (an autocrine mechanism of feedback regulation). The possibility that internalization might be a necessary step initially found many adherents, but it became apparent that the transduction mechanism emanates from events at the plasma membrane. In particular, ligand binding directly induces phosphorylation (on tyrosine residues) of a membrane protein, later shown to be the EGF receptor (EGF-R) itself (Ushiro and Cohen, 1980). This was an important breakthrough because tyrosine kinase activity had already been associated with virus-induced sarcomas (the v-src gene product). Thus, a firm link was established between neoplasia and the physiological regulation of cellular growth.

Further evidence for the role of growth factors in tumor generation came with the revelation that the avian erythroblastosis virus oncogene, v-erb-B, codes a product having similarities with the EGF receptor. Indeed, it became apparent that the transformation derives from inappropriate acquisition from the (cellular) c-erb-B gene, of a truncated receptor, lacking the binding site for EGF and which is constitutively activated (Downward et al., 1984).

The development of this field has generated much excitement but also frustration. It has been exciting, because it has yielded a good understanding of cell transformation and growth factors, and frustrating because it has become clear that cancer cells do not readily lend themselves as specific targets for drugs. The main impetus behind these studies was that nonmammalian genes might be the cause of disease. The hope was to discover targets that might be exploited to kill tumor cells selectively, for example, the products of the viral genes. We now realize that these are initially hijacked from the mammalian genome itself, then inaccurately transcribed by sloppy DNA or RNA polymerases in the virus, which then offers them back to the host upon infection. Apart from this, the large proportion of human tumors is of nonviral origin, arising as a consequence of tumor-promoting substances, radiation, etc.

Platelet-derived growth factor

In 1912, Alexis Carrel (Ross and Vogel, 1978) reported a number of experiments having the purpose:

> to determine the conditions under which the active life of a tissue outside that of the organism could be prolonged indefinitely

When he tried to maintain tissues for a few days in a simple buffered salts solution, the cells lost their growth capacity and then their viability. It was supposed that the senility and death of the cultures were due to the accumulation of catabolic substances and exhaustion of essential nutrients. Continuous and more rapid growth was achieved by supplementing the solution with diluted plasma, and then, from time to time, submerging the tissue in serum for a few hours. Interestingly, the notion that the cells might require specific factors present in the serum never appears to have crossed Carrel's mind. In conclusion, he wrote that

> fragments of connective tissue have been kept in vitro in a condition of active life for more than two months. As a few cultures are now eighty-five days old and are growing very actively, it is probable that, if no accident occurs, the life of these cultures will continue for a long time (Figure 1-15). ***Hamilton (1986)***

Alexis Carrel, the first American to be awarded the Nobel Prize 1912 "in recognition of his work on vascular suture and the transplantation of blood vessels and organs." He tarnished his reputation by his association with the eugenics movement, calling for the establishment of institutions equipped with "appropriate gases" in order to eliminate the insane. He gave enthusiastic support to the Vichy government during World War II and after the liberation of France he was charged for collaboration with the Nazi occupiers. He died before his case came to trial. Even his claim to have kept heart cells in culture may have been unwittingly fraudulent. Assistants may have introduced fresh cells from time to time in order to achieve the expected result…and to keep Alexis happy: see p. 21 of Hamilton (1986).

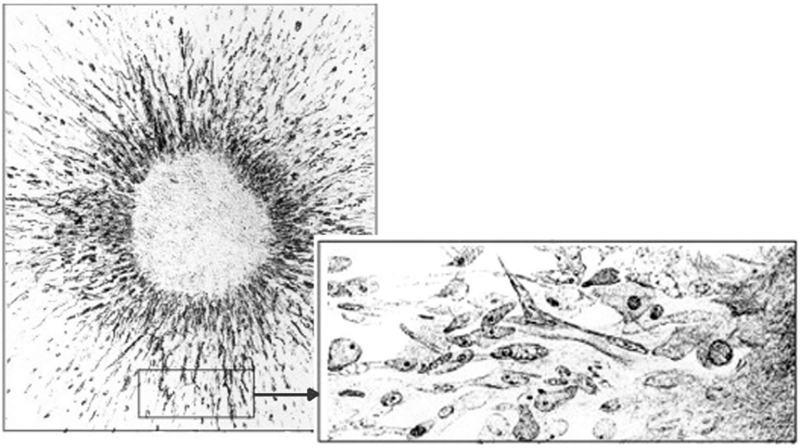


FIGURE 1-15 A 50-day-old culture of connective tissue. "Active tissue is encircling a piece of old plasma." *From* Carrel (1912).

About 40 years later, Temin and Dulbecco, working independently, set out to define the precise requirements for cell culture with respect to amino acids, vitamins, salts (together, Carrel's "nutrients"), and importantly, growth factors. Their ambition to culture cells arose from their interest in the role of viruses in cell transformation and tumor formation. Thus an important landmark was the finding that the requirement for serum is drastically reduced in cells infected with tumor viruses. They proposed that transformation might occur as a result of the enhanced capacity of tumor cells to respond to the proliferation signals present in the serum.

Howard Temin and **Renato Dulbecco** shared the Nobel Prize in 1975 for their discoveries concerning the interaction between tumor viruses and the genetic material of the cell.

An important turning point was the discovery that serum (the soluble component of *clotted* blood) supports growth and proliferation. Plasma (the soluble component of blood after sedimentation of cells) merely allows survival and over a period of about 2 days, cells become quiescent (arrested at the G0 stage of the cell cycle). Serum contains the products of activated platelets suggesting that they might have a necessary role in the provision of growth factors. In 1974, Russell Ross showed that factors extracted from platelets can induce quiescent smooth muscle cells to synthesize DNA (Ross et al., 1974), and in the same year, Kohler and Lipton obtained a similar result with mouse 3T3 fibroblasts. A purified factor derived from blood platelets, hence named platelet-derived growth factor (PDGF), could propel the quiescent cells into the cell cycle S-phase. With the purification of PDGF (Antoniades et al., 1979) the question of how it acts to could be faced. PDGF exists as a disulfide linked dimer, so that binding automatically causes the cross-linking of two receptors. This constitutes the signal for activation (Cooper et al., 1982). Subsequently, it was found that the oncogene of the simian sarcoma virus (v-sis) is homologous to the gene coding for PDGF (Doolittle et al., 1983; Waterfield et al., 1983; Robbins et al., 1983). Here was another clear link between a growth factor and a tumor virus. This time, however, the signal to uncontrolled cell proliferation is due to excessive production of growth factor, rather than expression of a constitutively activated receptor. Furthermore, it was shown that v-sis causes cell transformation in primates (Theilen et al., 1971).

The mechanisms by which an oncogene might cause a tumor were becoming clearer. Here is another example of a mammalian gene, surreptitiously borrowed by a virus, then mutated or mutilated. On return to the host by infection, it causes cell transformation and tumor formation.

Transforming growth factors TGFα and TGFβ

The transforming growth factors (TGF) were originally isolated from the conditioned medium of a virally infected mammalian fibroblast cell line, 3T3. These are proteins that can bring about transformation of phenotype (Figure 1-16). The discovery of the TGF followed some years after the first reports and descriptions of EGF and it derives particularly from the undertakings in the laboratory of George Todaro (De Larco and Todaro, 1978).

CELL TRANSFORMATION

Fully transformed cells possess one or more of the following characteristics: they may not require exogenous growth factors, they grow in athymic (nude) mice, they are insensitive to contact-inhibition, they can evade programmed cell death (apoptosis), they have limitless replicative potential, they can induce angiogenesis, they no longer require attachment to a substrate (they grow in soft agar), and they may disseminate in different tissues (metastasis).

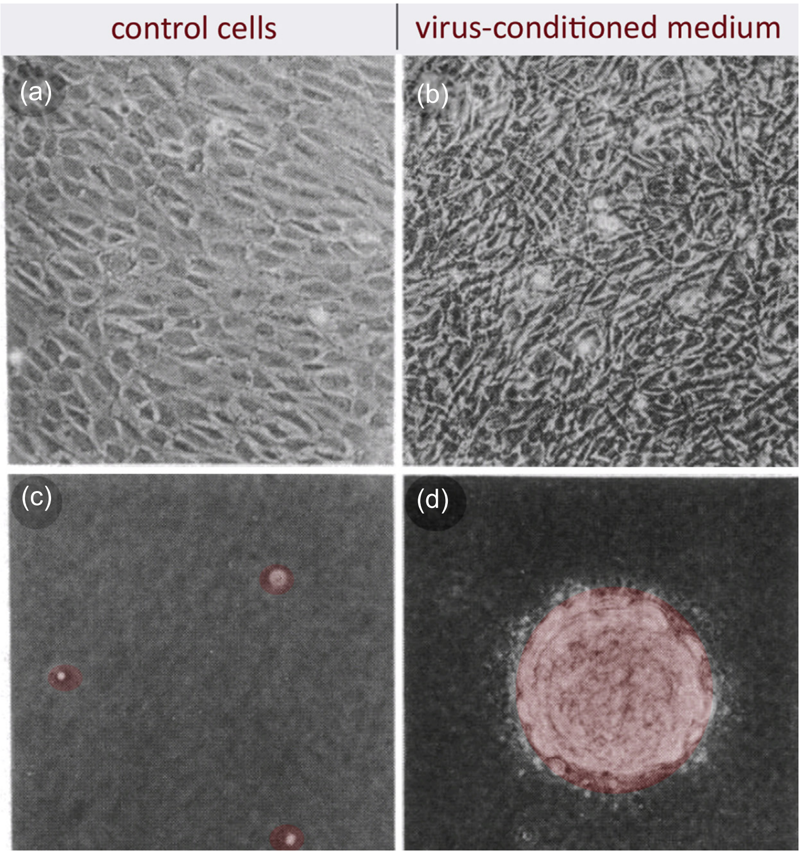


FIGURE 1-16 Cell transformation through sarcoma virus-derived growth factors. Fibroblasts cells were brought into culture with (b and d) or without (a and c) medium obtained from sarcoma virus-infected cells (virus-conditioned medium). Note that with conditioned medium (b) cells are no longer contact inhibited and grow in multiple layers and (d) they form colonies in soft agar.

TGFα, although quite distinct from EGF with respect to amino acid sequence, binds to the EGF receptor and signal cells in a similar fashion (see Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases"). A related factor isolated from these tumor cells, TGFβ, which does not compete with the binding of TGFα or EGF, can nevertheless induce transformation when provided with either of these two factors. Importantly, TGFβ is a normal cellular product and the finding of high quantities in blood platelets and its release during blood coagulation established a clear link with PDGF (Childs et al., 1982).

In screening the transforming effect of TGFβ in numerous tumors, there was an unexpected finding. Depending on the cells and the conditions, TGFβ can either promote or suppress cell growth and transformation. It cooperates with TGFα and EGF to cause cell transformation. On the other hand, it inhibits colony formation in cells derived from human tumors. It appears that its effects are a function of the total set of growth factors and their receptors that are operational at a given time (Roberts et al., 1985). In addition, TGFβ plays a number of key roles in the process of tissue remodeling and wound healing (Sporn et al., 1987). It induces the production of fibronectin and collagen and thus regulates the deposition of the cell matrix, itself a key determinant of cell growth (see Chapter 17).

PROBLEMS WITH NOMENCLATURE

As must be evident, nomenclature in this area is arbitrary, to say the least. Some growth factors were named after the cells from which they were first isolated, others from the cells which they stimulated, yet others from the principal action that they appeared to perform (Roberts et al., 1980). In immunology, we hear of interleukins and colony-stimulating factors. These direct the maturation and proliferation of white blood cells. In virology, we have the interferons that "interfere" with viral infection and in cancer research we have tumor necrosis factor (and its relatives) that can influence the growth of solid tumors. In each discipline, it seemed that these factors functioned mainly in the category in which they first came to light. Of course, we now know that some growth factors have actions that are totally unrelated to growth. For instance, PDGF, released from platelets at sites of tissue damage (Kaplan et al., 1979), not only supports the growth of fibroblasts, smooth muscle cells, and glial cells, but also acts to regulate the distribution and migration of vascular smooth muscle cells and fibroblasts in wound healing (Grotendorst et al., 1982).

To add further complexity to an already complex situation, the conditions in which the cells are studied can determine the cellular response, for instance, the presence of other factors, other cells, attachment to substrates. A good example of this is TGF-β. As its name implies, this emerged as a factor enhancing cell transformation, but we now recognize that it can inhibit cell proliferation (Cone et al., 1988) and that it is a very potent chemotactic factor for neutrophils (Haines et al., 1993) and fibroblasts (Postlethwaite et al., 1987). It has been proposed that a common name for these factors should be cytokines (Nathan and Sporn, 1991). While offering no clues to their various actions, this definition represents a move toward coordinating our understanding of their roles as first messengers. The unity of the cytokines is a concept as important as that of the hormones, defined by Bayliss and Starling nearly 90 years ago.

Cytokines: Soluble (glyco)proteins, non-immunoglobulin in nature, released by living cells of the host, acting nonenzymatically in picomolar to nanomolar concentrations to regulate host cell function.

References

Adjalley, S., Johnston, G.L., Li, T., Eastman, R.T., Ekland, E.H., Eappen, A.G., Richman, A., Sim, K.L., Lee, M.C.S., Hoffman, S.L., Fidock, D.A., 2011. Quantitative assessment of *Plasmodium falciparum* sexual development reveals potent transmission-blocking activity by methylene blue. PNAS USA 108, 18867–18868.

Aminoff, M.J., 1993. Brown-Séquard: A Visionary of Science. Raven Press, New York.

Anantharaman, V., Iyer, L.M., Aravind, L., 2007. Comparative genomics of protists: new insight into the evolution of eukaryotic signal transduction and gene regulation. Annu. Rev. Microbiol. 61, 453–475.

Antoniades, H.N., Scher, C.D., Stiles, C.D., 1979. Purification of human platelet-derived growth factor. Proc. Natl. Acad. Sci. U.S.A. 76, 1809–1813.

Bacq, Z.M., 1975. Chemical Transmission of Nerve Impulses: An Historical Sketch. Pergamon, Oxford.

Baker, M.E., 2003. Evolution of adrenal and sex steroid action in vertebrates: a ligand-based mechanism for complexity. Bioessays 25, 396–400.

Bayliss, W.M., 1924. Principles of General Physiology. Longmans, London, p. 739.

Bennet, M.R., 2000. The concept of transmitter receptors: 100 years on. Neuropharmacology 39, 523–546.

Bilski, R., Kaulbersz, J., 1987. Napoleon cybulski, 1854–1919. Acta Physiol. Pol. 38, 74–90.

Borrell, M., 1976. Organotherapy, British physiology and the discovery of internal secretions. J. Hist. Biol. 9, 236–268.

Bray, D., 2009. Wetware: A Computer in Every Living Cell. Yale University Press, New Haven, Connecticut.

Bren, A., Eisenbach, M., 2000. How signals are heard during bacterial chemotaxis: protein-protein interactions in sensory signal propagation. J. Bacteriol. 182, 6865–6873.

Brown-Séquard, C.E., 1889a. The effects produced on man by subcutaneous injections of a liquid obtained from the testicles of animals. Lancet 2, 105–107.

Brown-Séquard, C.E., 1889b. Seconde note sur les effets produits chez l'homme par les injection sous-cutanées d'un liquide retiré des testicules frais de cobaye et de chien. C. R. Soc. Biol. Paris 41, 420.

Bueker, E.D., 1948. Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbosacral nervous system. Anat. Rec. 102, 369–389.

Camporesi, P., 1989. Bread of dreams. Hist. Today 39, 14–21.

Cannon, W.B., 1929. Bodily Changes in Pain, Hunger, Fear and Rage. An Account of Researches into the Function of Emotional Excitement. Harper Torch Books, New York. Reprinted 1963.

Carrel, A., 1912. On the permanent life of tissues outside of the organism. J. Exp. Med. 516–528.

Cerione, R.A., Sibley, D.R., Codina, J., Benovic, J.L., Winslow, J., Neer, E.J., Birnbaumer, L., Caron, M.G., Lefkowitz, R.J., 1984. Reconstitution of a hormone-sensitive adenylate cyclase system. The pure beta-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. J. Biol. Chem. 259, 9979–9982.

Changeux, J.-P., Kasai, M., Lee, C.Y., 1970. The use of a snake venom toxin to characterize the cholinergic receptor protein. Proc. Natl. Acad. Sci. U.S.A. 67, 1241–1247.

Childs, C.B., Proper, J.A., Tucker, R.F., Moses, H.L., 1982. Serum contains a platelet-derived transforming growth factor. Proc. Natl. Acad. Sci. U.S.A. 79, 5312–5316.

Cohen, S., Levi-Montalcini, R., 1958. A nerve-growth stimulating factor isolated from snake venom. Proc. Natl. Acad. Sci. U.S.A. 42, 571–574.

Cohen, S., 1964. The stimulation of epidermal proliferation by a specific protein (EGF). Dev. Biol. 12, 394–407.

Cohen, S., 1986. Nobel lecture. Epidermal growth factor. Biosci. Rep. 6, 1017–1028.

Cone, J.L., Brown, D.R., DeLarco, J.E., 1988. An improved method of purification of transforming growth factor, type β from platelets. Anal. Biochem. 168, 71–74.

Cooper, J.A., Bowen, P.D., Raines, E., Ross, R., Hunter, T., 1982. Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. Cell 31, 263–273.

Croonian lecture. Proc. R. Soc. Lond. 66, 1900, 433–434.

Dakin, H.D., 1905. The synthesis of a substance allied to adrenalin. Proc. R. Soc. Lond. B 76, 491–497.

Dale, H.H., 1948. Accident and opportunism in medical research. Brit. Med. J. 2, 451–455.

Dale, H.H., 1953. Adventures in Physiology. Pergamon, London.

Davenport, H., 1991. Early history of the concept of chemical transmission of the nerve impulse. Physiologist 34, 129–139.

De Larco, J.E., Todaro, G.J., 1978. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. U.S.A. 75, 4001–4005.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G., Lefkowitz, R.J., Strader, C.D., 1986. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature 321, 75–79.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., Antoniades, H.N., 1983. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275–277.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., Waterfield, M.D., 1984. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307, 521–527.

Dyson, F., 1999. Origins of Life. Cambridge University Press, New York.

Editorial annotation, 1889. The new elixir of youth. Brit. Med. J. 1, 1416.

Editorial annotation, 1925. Can old age be deferred? an interview with Dr Serge Voronoff, the famous authority on the possibilities of gland transplantation. Sci. Am. 226–227.

Ellermann, V., Bang, O., 1908. Experimentelle leukämie bei hünern. Zentr Bakteriol. Parasitenk 4, 595–609.

Elliott, T.R., 1905. The action of adrenaline. J. Physiol. 32, 401–467.

Erikson, R.L., Purchio, A.F., Erikson, E., Collett, M.S., Brugge, J.S., 1980. Molecular events in cells transformed by Rous Sarcoma virus. J. Cell Biol. 87, 319–325.

Escriva, H., Delaunay, F., Laudet, V., 2003. Ligand binding and nuclear receptor evolution. Bioessays 22, 717–727.

Fletcher, W.M., 1926. Obituary notice of John Newport Langley. J. Physiol. 61, 1–27.

Goodman, L.S., Gilman, A., 1955. The Pharmacological Basis of Therapeutics.

Grand, S., 2001. Creation, Life and How to Make it. Harvard University Press, Cambridge, Massachusetts.

Gregory, H., 1975. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature 257, 325–327.

Grotendorst, G.R., Chang, T., Seppa, H.E., Kleinman, H.K., Martin, G.R., 1982. Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell Physiol. 113, 261–266.

Haines, K.A., Kolasinski, S.L., Cronstein, B.N., Reibman, J., Gold, L.I., Weissmann, G., 1993. Chemoattraction of neutrophils by substance P and transforming growth factor-β 1 is inadequately explained by current models of lipid remodeling. J. Immunol. 151, 1491–1499.

Hamburger, V., Levi-Montalcini, R., 1949. Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111, 457–502.

Hamburger, V., 1969. Hans Spemann and the organizer concept. Experientia 25, 1121–1125.

Hamilton, D., 1986. The Monkey Gland Affair. Chatto and Windus, London.

Hunt, R., Taveau, R.D, 1906. On the physiological action of certain choline derivatives and new methods for detecting choline. Brit. Med. J. 2, 1788–1791.

Hunt, L.T., Dayhoff, M.O., 1979. Structural and functional similarities among hormones and active peptides from distantly related eukaryotes. In: Gross, E., Meienhofer, J. (Eds.), Peptides: Structure and Biological Function. Pierce Chemical Co, Rockford.Il, pp. 757–760.

Hunter, T., Sefton, B.M., 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77, 1311–1315.

Huxley, T.H., 1849. On the anatomy and the affinities of the family of the Medusae. Philos. Trans. R. Soc. Lond. 139, 413–434. Published by: The Royal Society, obtained through JSTOR http://www.jstor.org/stable/108486.

Hildebrand, E., 1977. What does Halobacterium tell us about photoreception? Biophys. Struct. Mech. 3, 69–77.

Janakidevi, K., Dewey, V.C., Kidder, G.W., 1966a. Serotonin in protozoa. Arch. Biochem. Biophys. 113, 758–759.

Janakidevi, K., Dewey, V.C., Kidder, G.W., 1966b. The biosynthesis of catecholamines in two genera of protozoa. J. Biol. Chem. 241, 2576–2578.

Jenkinson, D.H., 2011. Classical approaches to the study of drug-receptor interactions. In: Textbook of Receptor Pharmacology. CRC Press, Boca Raton, Florida, pp. 3–78.

Kaplan, D.R., Chao, F.C., Stiles, C.D., Antoniades, H.N., Scher, C.D., 1979. Platelet α granules contain a growth factor for fibroblasts. Blood 53, 1043–1052.

Katz, B., Miledi, R., 1970. Membrane noise produced by acetylcholine. Nature 226, 962–963.

Kenny, J.J., Martinez, M.O., Fehniger, T., Ashman, R.F., 1979. Lipid synthesis: an indicator of antigen-induced signal transduction in antigen-binding cells. J. Immunol. 122, 1278–1284.

Koman, A., Harayama, S., Hazelbauer, G.L., 1979. Relation of chemotactic response to the amount of receptor: evidence for different efficiencies of signal transduction. J. Bacteriol. 138, 739–747.

Lam, H.-M., Chiu, J., Hsieh, M.-H., Meisel, L., Oliviera, I.C., Shin, M., Coruzzi, G., 1998. Glutamate-receptor genes in plants. Nature 396, 127–128.

Langley, J.N., 1878. On the physiology of the salivary secretion. Part II. On the mutual antagonism of atropin and pilocarpin have especial reference to their relations in the submaxillary gland of the cat. J. Physiol. I, 339–369.

Langley, J.N., 1905. On the reaction of cells and of nerve endings to certain poisons chiefly as regards the reaction of striated muscle to nicotine and to curare. J. Physiol. 33, 374–413.

LeRoith, D., Shiloach, J., Roth, J., Lesniak, M.A., 1980. Evolutionary origins of vertebrate hormones: substances similar to mammalian insulins are native to unicellular eukaryotes. Proc. Natl. Acad. Sci. U.S.A. 77, 6184–6188.

LeRoith, D., Shiloach, J., Roth, J., Liotta, A.S., Krieger, D.T., Lewis, M., Pert, C.B., 1981. Evolutionary origins of vertebrate hormones: material very similar to adrenocorticotropic hormone, β-endorphin, and dynorphin in protozoa. Trans. Assoc. Am. Physicians 94, 52–60.

LeRoith, D., Liotta, A.S., Roth, J., Shiloach, J., Lewis, M.E., Pert, C.B., Krieger, D.T., 1982. ACTH and β-endorphin-like materials are native to unicellular organisms. Proc. Natl. Acad. Sci. U.S.A. 79, 2086–2090.

LeRoith, D., Shiloach, J., Berelowitz, M., Frohman, L.A., Liotta, A.S., Krieger, D.T., Roth, J., 1983. Are messenger molecules in microbes the ancestors of the vertebrate hormones and tissue factors? Fed. Proc. 42, 2602–2607.

Levi-Montalcini, R., 1950. The origin and development of the visceral system in the spinal cord of the chick embryo. J. Morphol. 86, 253–283.

Levi-Montalcini, R., 1988. In: Praise of Imperfection, My Life and Work (Translation by Attardi, L. of Elogio dell'imperfezione). Basic Books, New York.

Levi-Montalcini, R., 1997. The Saga of the Nerve Growth Factor: Preliminary Studies, Discovery, Further Development. World Scientific Publishing Co, Singapore.

Loewi, O., 1960. An autobiographical sketch. Perspect. Biol. Med. 4, 3–25.

Maehle, A.-H., Prull, C.-R., Halliwell, R.F., 2002. The emergence of the drug receptor theory. Nat. Rev. Drug Discov. 1, 637–641.

Manzanares, M., Wada, H., Itasaki, N., Krumlauf, R., Holland, P.W.H., 2003. Conservation and elaboration of Hox gene regulation during evolution of the vertebrate head. Nature 418, 854–857.

Medvei, V.C., 1993. The History of Clinical Endocrinology. The Parthenon Publishing Group Ltd, Carnforth, Lancs. UK.

Meier, A.H., 1977. Daily rhythms of lipogenesis in fat and lean white-throated sparrows *Zonotrichia albicollis*. Am. J. Physiol. 232, E193–E196.

Meier, A.H., Farner, D.S., King, J.R., 1965. A possible endocrine basis for migratory behaviour in the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. Anim. Behav. 13, 453–465.

Miledi, R., Molinoff, P., Potter, L.T., 1971. Isolation of the cholinergic receptor protein of Torpedo electric tissue. Nature 229, 554–557.

Moore, B.M., 1895. On the chemical nature of a physiologically active substance occurring in the suprarenal gland. Proc. Physiol. Soc. 17, xiv–xvii.

Murray, G.R., 1891. Note on the treatment of myxoedema by hypodermic injections of an extract of the thyroid gland of a sheep. Brit. Med. J. 2, 796–797.

Nathan, C., Sporn, M., 1991. Cytokines in context. J. Cell Biol. 113, 981–986.

Nicoll, C.S., 1982. Prolactin and growth hormone: specialists on one hand and mutual mimics on the other. Perspect. Biol. Med. 25, 369–381.

Nobel Speech AG Gilman, 1994.

Oliver, G., Schäfer, E.A., 1894. On the physiological action of extract of the suprarenal capsules. Proc. Physiol. Soc. 9, i–iv.

Oliver, G., Schäfer, E.A., 1895. On the physiological action of extract of the suprarenal capsules. Proc. Physiol. Soc. 17, ix–xiv.

"Paul Ehrlich - Nobel Lecture: Partial Cell Functions". Nobelprize.org. Nobel Media AB 2014.

Postlethwaite, A.E., Keski Oja, J., Moses, H.L., Kang, A.H., 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J. Exp. Med. 165, 251–256.

Prabhu, Y., Eichinger, L., 2006. The Dictyostelium repertoire of seven transmembrane domain receptors. Eur. J. Cell Biol. 85, 937–946.

Ramamoorthy, K., Shanker Verma, R., 2008. Convergence pattern studies of folate receptor. Bioinformation 3 (4), 168–172.

Rang, H.P., Dale, M.M., Ritter, J.M., Flower, R.J., 2007. Pharmacology, sixth ed. Churchill Livingstone/Elsevier. pp. 131–188.

Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W., Aaronson, S.A., 1983. Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature 305, 605–608.

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., De Larco, J.E., Todaro, G.J., 1980. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. U.S.A. 77, 3494–3498.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F., Sporn, M.B., 1985. Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. U.S.A. 82, 119–123.

Rodbell, M., 1980. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature 284, 17–22.

Rous, P., 1910. A transmissible avian neoplasm. (Sarcoma of the common fowl). J. Exp. Med. 12, 696–705.

Rous, P., 1967. Comment. Proc. Natl. Acad. Sci. U.S.A. 58, 843–845.

Ross, R., Vogel, A., 1978. The platelet-derived growth factor. Cell 14, 203–210.

Ross, R., Glomset, J., Kariya, B., Harker, L., 1974. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. U.S.A. 71, 1207–1210.

Sakula, A., 1882. Robert Koch: centenary of the discovery of the tubercle bacillus. Can. Vet. J. 1983 (24), 127–131.

Schaap, P., 2011. Evolution of developmental cyclic adenosine monophosphate signaling in the Dictyostelia from an amoebozoan stress response. Dev. Growth Differ. 53 (4), 452–462.

Schäfer, E.A., 1908. Present condition of our knowledge regarding the functions of the suprarenal capsules. Brit. Med. J. 1, 1277–1281.

Shen, W.-M., Salemi, B., Will, P., 2002. Hormone-inspired adaptive communication and distributed control for CONRO self-reconfigurable robots. IEEE Trans. Rob. Autom. 18, 700–712.

Shen, W.-M., Will, P., Galstyan, A., Chuong, C.-M., 2004. Hormone-inspired self-organization and distributed control of robotic swarms. Auton. Robots 17, 93–105.

Short, R.V., 1999. Did Parisians catch HIV from “monkey glands”? Nature 398, 657.

Skorokhod, A., Gamulin, V., Gundacker, D., Kavsan, V., Muller, I.M., 1999. Origin of insulin receptor-like tyrosine kinases in marine sponges. Biol. Bull. 197, 198–206.

Spiegel, F., 1996. Sick Notes: An Alphabetical Browsing Book of Derivations, Abbreviations, Mnemonics and Slang for the Amusement and Edification of Medics, Nurses, Patients and Hypochondriacs. The Parthenon Publishing Group Ltd, Carnforth, Lancashire, UK.

Sporn, M.B., Roberts, A.B., Wakefield, L.M., de-Crombrugghe, B., 1987. Some recent advances in the chemistry and biology of transforming growth factor-β. J. Cell Biol. 105, 1039–1045.

Springer, M.S., Goy, M.F., Adler, J., 1979. Protein methylation in behavioural control mechanisms and in signal transduction. Nature 280, 279–284.

Starling, E.H., 1905. On the chemical correlations of the functions of the body. Lancet 2, 339–341.

Stolz, F., 1904. Über adrenalin und alkylaminoacetobrenzcatechin. Ber. Deutch Chem. Ges. 37, 4149–4154.

Theilen, G.H., Gould, D., Fowler, M., Dungworth, D.L., 1971. C-type virus in tumor tissue of a woolly monkey (Lagothrix spp.) with fibrosarcoma. J. Natl. Cancer Inst. 47, 881–889.

Ushiro, H., Cohen, S., 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J. Biol. Chem. 255, 8363–8365.

Vesely, D.L., Giordano, A.T., 1992. Atrial natriuretic factor-like peptide and its prohormone within single cell organisms. Peptides 13, 177–182.

Vesely, D.L., Gower Jr., W.R., Giordano, A.T., 1993. Atrial natriuretic peptides are present throughout the plant kingdom and enhance solute flow in plants. Am. J. Physiol. 265, E465–E477.

Wainwright, M., 2010. Dyes, trypanosomiasis and DNA: a historical and critical review. Biotech. Histochem. 85, 341–354.

Ward, T.H., Polishchuk, R.S., Caplan, S., Hirschberg, K., Lippincott-Schwartz, J., 2001. Maintenance of Golgi structure and function depends on the integrity of ER export. J. Cell Biol. 155, 557–570.

Warthin, A.S., 1904. Trans. Assoc. Am. Physicians 19, 421.

Warthin, A.S., 1907. Leukemia of the common fowl. J. Infect. Dis. 4, 369–380.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J.S., Deuel, T.F., 1983. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature 304, 35–39.

Yeo, G.F., 1889. A Manual of Physiology, fourth American ed. P Blakiston, Son & Co., Philadelphia.

CHAPTER

2

An Introduction to Signal Transduction

CELLS NEED WAYS TO CREATE SYMBOLIC REPRESENTATIONS OF THEIR (CHANGING) ENVIRONMENT

> A goal for the future would be to determine the extent of knowledge the cell has of itself and how it utilizes this knowledge in a "thoughtful" manner when challenged.
> ***Barbara McClintock. Nobel Prize acceptance lecture, 1983***

The above quote relates to homeostatic adjustments required of cells, and McClintock refers to the sensing devices that signal adjustments in the number of ribosomal DNA loci, mishaps affecting DNA, or amitotic division. We refer to this as the "signals from within," such as those that recruit nucleotide repair machinery to the DNA or allow cells to deal with protein folding stress in the rough endoplasmic reticulum. Knowing their inner self is one thing, but cells also need to deal with their environment and this is particularly complex when they are part of an organism. Importantly, the wellbeing of the organism impacts on that of the cells and good lines of communication between the two entities are primordial. Taking this into account we can add a second scientific goal by adjusting the quote of McClintock as follows:

> A goal for the future will be to determine the extent of knowledge the cell has of its environment and how it utilizes this knowledge in a « thoughtful » manner when the organism, in which it takes part, is challenged.

Cells need ways to create symbolic representations of their (changing) environment and how they do this is the subject of our interest. We take an anthropocentric approach and will relate much of our knowledge to the functioning of human cells. We certainly deal with frogs, flies, and worms but within the context of how these organisms have contributed to the understanding of cell functioning in man (male and female). For

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multicellular organisms, the cellular environment can be divided into two compartments: the outside and the inside. With respect to the outside, sense organs such as the skin, ears, tongue, nose, and eyes detect the presence of light, sound waves, gravity, motion, flavors, odorants, pressure, temperature, and others. These specialized structures signal environmental clues in the form of action potentials into the body in order to be interpreted by the brain. The lumen of the digestive tract qualifies *stricto sensu* as the outside (remember, you can pass a thread from mouth to anus) but it nevertheless has a distinct approach to sensing its environment (presence of bacteria and food), in part because of a totally different organization of the epithelium. Concerning the inside, here we deal with an environment composed of cells and their matrix (basement membrane, connective tissue, cartilage, bone, and plasma). How cells perceive and deal with this environment depends on the receptors they express and the substances they secrete. Perception of the inside employs few specialized sense structures, examples of which are muscle spindles and Golgi-tendon organs. They are "proprioceptors" that provide information about joint angle, muscle length, and tendon tension. Proprioceptor is an amalgamation of two Latin words, *proprius* (one's own) and *perception*. They signal through action potentials. It should be noted that certain areas in the brain could qualify as specialized sense structures. The one that comes to mind is the arcuate nucleus, which receives signals about the feeding state of the organism. However, we leave this subject to be dealt with by other experts. Below we describe how cells perceive their environment, and because our emphasis lies on the internal environment, in essence we describe of how cells communicate with one another (Figure 2-1).

WETWARE

The quote from McClintock also features in the book "Wetware" by Denis Bray (from the University of Oxford). "Wetware" refers to cellular software as well as hardware. Cells do not have electronic circuits, but they do have circuits of biomolecules, in which proteins play a major role, and these can vary in intensity (concentration) and composition as a consequence of a multitude of posttranslational modifications or interactions with small molecules and other proteins. An analogy with computers, or more precisely with (dynamic) 3D-integrated circuits (systems-on-chip), is certainly in place although we still need to be cautious as we are only beginning to learn how cells integrate the multiple signals that arrive at any time (Bray, 2009). Here lies one of the difficulties for science writing. Metaphors are often needed to pass on a clear message to non experts (students and laymen). They also help in obtaining big funding or sympathy

WETWARE *(cont'd)*

for the scientific effort. Metaphors contribute to the sense-making process but at the same time they often lead to tenacious misconceptions due to inappropriate simplifications. The human genome project, and its promise to obtain the "blueprint" for how human beings are made (and therefore cure genetic-based diseases like cancer), is a case in point (Pigliucci and Boudry, 2011).

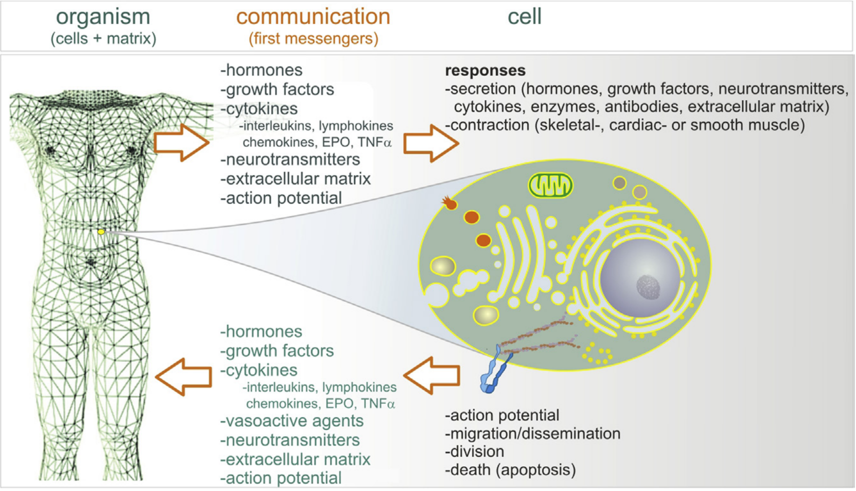


FIGURE 2-1 Communication between the cell and the organism.

FIRST MESSENGERS

With the exception of action potentials traveling along nerve cells, communication between cells basically occurs through secreted substances that we call first messengers. They may appear under a variety of generic names such as agents, mediators, ligands, humoral factors, or communication medium. First messengers come in a great variety, with their chemical composition ranging from gases, single amino acids, nucleotides, lipids to large proteins. When they are produced by cells organized in specialized tissues, the so-called glands, they communicate in an endocrine fashion. When they originate from ordinary tissue cells (not glands), they communicate in a paracrine fashion. When the diffusion of the first messenger is limited to a synaptic cleft, they act in synaptic transmission. When the first messenger remains attached to the cell, they communicate in a juxtacrine fashion (Figure 2-2). Finally, when the secreting cells express the appropriate receptor, an

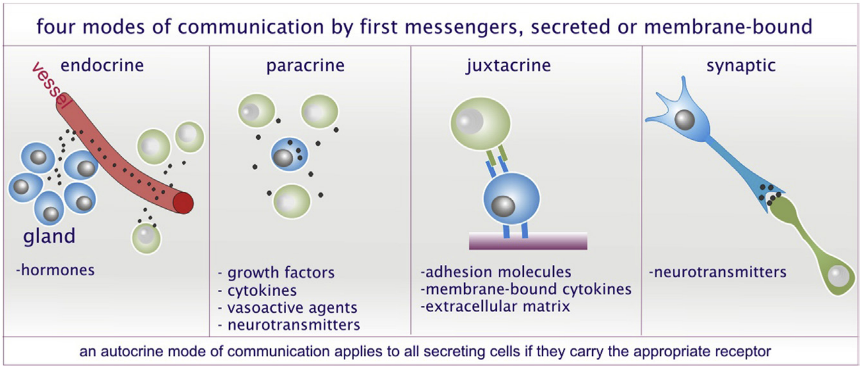

four modes of communication by first messengers, secreted or membrane-bound
endocrine
vessel
gland
-hormones
paracrine
- growth factors
- cytokines
- vasoactive agents
- neurotransmitters
juxtacrine
-adhesion molecules
-membrane-bound cytokines
-extracellular matrix
synaptic
-neurotransmitters
an autocrine mode of communication applies to all secreting cells if they carry the appropriate receptor

FIGURE 2-2 Four modes of communication by first messengers.

autocrine mode of communication applies to all of these settings. It should be understood that the anatomical boundary of paracrine communication is extremely variable. A strong local production of inflammatory mediators has systemic effects, reaching the whole body, and makes us feel ill.

The subdivision of first messengers is not very systematic. This is due in part because of their progressive discovery (the list is still growing with the systematic screening of the various genomes) and because they have a much less restricted mode of action than was initially anticipated. We speak of hormones, growth factors, neurotransmitters, neuromodulators, interleukins (IL), lymphokines, inflammatory mediators, and chemoattractants. Each of these terms attempts to define a class of agents that take effect in a particular setting but we now know that none of them adheres to a strict definition. As we have mentioned in Chapter 1, the term cytokine has been proposed for all mediators, of protein origin, comprising growth factors, certain inflammatory mediators, and lymphokines (IL, GM-CSF, and IFN), but this has not been universally adopted. Table 2-1 provides examples of first messengers. Table 2-2 lists a selection of substances that can act as neurotransmitters, note the diversity in chemical composition.

Particular examples of multiple functions are:

- The nucleotide ATP and the amino acid glutamate are ordinary cellular metabolites but act as neurotransmitters when they are secreted at synapses.
- The hormones gastrin, cholecystokinin, and secretin originally discovered in gut cells are also produced in the central nervous system (CNS), where they have diverse functions as neuromodulators (influencing the release of other neurotransmitters).
- Somatostatin, identified originally as a hypothalamic agent suppressing the secretion of growth hormone (GH) from the pituitary, also operates in the CNS as a neurotransmitter or neuromodulator. More than this, it is a paracrine agent in the pancreas and a hormone for the liver.

TABLE 2-1 A selection of first messengers found in the blood circulation

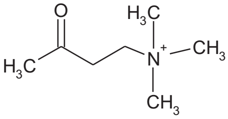
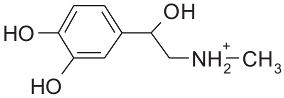
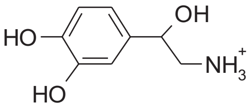
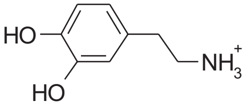
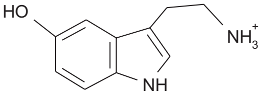
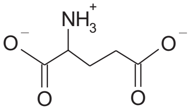
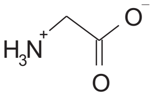
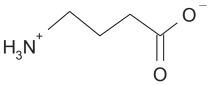
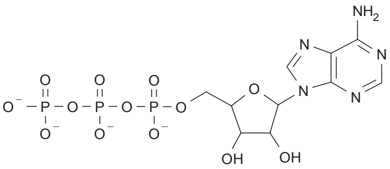
Class	Messenger	Origin	Target	Major effects
Messengers derived from amino acids	Adrenaline (epinephrine)	Adrenal medulla	Heart smooth muscle, liver, and muscle adipose tissue	Increase in pulse rate and blood pressure contraction or dilation glycogenolysis lipolysis
	Noradrenaline (norepinephrine)	Adrenal medulla	Arteriolar smooth muscle	Vasoconstriction
	Serotonin (5-hydroxy tryptamine)	Platelets	Arterioles and venules	Vasodilation and increased vascular permeability
	Histamine	Mast cells, basophils	Arterioles and venules	Vasodilation and increased vascular permeability
	Thyroid hormone, T3 (tri-iodothyronine)	Thyroid	Multiple	Metabolic regulation
Peptide hormones	Insulin	Pancreatic β-cells	Multiple tissues	Glucose uptake into cells carbohydrate catabolism protein synthesis lipid synthesis (adipose tissue) Note also a growth factor
	Glucagon	Pancreatic α-cells	Liver adipose tissue	Glycogenolysis lipolysis
	Gastrin	Intestine	Stomach	Secretion of HCI and pepsin
	Secretin	Small intestine	Pancreas	Secretion of digestive enzymes
	Cholecystokinin	Small intestine	Pancreas	Secretion of digestive enzymes
	Atrial natriuretic factor (ANF or ANP)	Heart	Kidney	Na^+ and water diuresis
	Adrenocorticotropic hormone (ACTH)	Anterior pituitary	Adipose tissue adrenal cortex	Lipolysis production of cortisol and aldosterone
	Follicle-stimulating hormone (FSH)	Anterior pituitary	Oocyte/ovarian follicles	Growth estrogen synthesis
	Luteinizing hormone (LH)	Anterior pituitary	Oocyte, ovarian follicles	Maturation, estrogen and progesterone synthesis
	Thyroid-stimulating hormone (thryotropin or TSH)	Anterior pituitary	Thyroid	Generation and release of thyroid hormones

Continued

TABLE 2-1 A selection of first messengers found in the blood circulation—cont'd

Class	Messenger	Origin	Target	Major effects
	Parathyroid hormone (PTH)	Parathyroid	Bone, kidney	Release of Ca^{2+} and phosphate, Ca^{2+} reabsorption
	Vasopressin (antidiuretic hormone or ADH)	Posterior pituitary	Kidney, arteriolar smooth muscle	Water reabsorption from urine, vasoconstriction to increase blood pressure
	Oxytocin	Posterior pituitary	Uterine smooth muscle, milk ducts	Cervical dilation, milk ejection
Growth factors (polypeptides)	Epidermal growth factor (EGF)	Multiple cell types	Epidermal and other cells	Cell proliferation
	Somatotropin (growth hormone or GH)	Anterior pituitary	Liver	Production of somatomedins
	Somatomedins 1 and 2 (insulin-like growth factors, IGF1 and 2)	Liver	Bone, muscle, and other cells	Cell proliferation
	Transforming growth factor β (TGFβ)	Multiple locations	Multiple	Cell proliferation and motility, matrix deposition
Eicosanoids (fatty acids)	Prostaglandins PGA_1,PGA_2, PGE_2	Most body cells	Multiple	Inflammation, vasodilation
Cytokines (polypeptides)	TNF-α interleukin 1b	Multiple cells macrophages	Production C-reactive protein, fever, cachexia, phagocytosis, lymphocytes	Proliferation, pyrogen
Cholesterol-derived hormones (steroids)	Progesterone	Corpus luteum placenta	Uterine endometrium	Preparation of endometrial layer, maintenance of pregnancy

TABLE 2-2 Neurotransmitters

Type	Transmitter	Structure
	Acetylcholine	
Tyrosine or tryptophan derived	Adrenaline (epinephrine)	
	Noradrenaline (norepinephrine)	
	Dopamine	
	Serotonin	
Amino acids	Glutamate	
	Glycine	
	GABA	
Purine based	ATP	
Neuropeptides	Enkephalins	Tyr–Gly–Gly–Phe–Leu Tyr–Gly–Gly–Phe–Met
	Substance P	Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met
	Angiotensin II	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
	Somatostatin	Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys

- The growth factor TGFβ modulates cell proliferation, regulates matrix deposition, acts as a chemoattractant, and induces cytoskeletal structures named podosomes (Varon et al., 2006).
- Insulin acts not only to regulate glucose metabolism but also as a growth factor.
- Noradrenaline (norepinephrine), depending on whether it is released at a synapse or from the adrenal medulla, can be considered as a neurotransmitter or as a hormone.
- Thrombin is a growth factor but is also involved in blood clotting, acting as an activator of platelet function.

Hormones

Hormones, meaning "I arouse" or "I excite," are the first discovered humoral factors involved in cell communication (see also Chapter 1, "Prologue"). They are commonly released in small amounts at glands remote from the organs they target. On entering the circulation, they are diluted enormously and are subject to enzymes that break them down. Many of them circulate as complexes with specific binding-proteins, reducing their free concentration still further. The result of all this is that their level in the vicinity of a target cell is likely to be extremely low and accordingly, the cell must possess high-affinity-binding sites (receptors) in order to be able to respond.

Examples of water-soluble hormones are included in the list of first messengers shown in Tables 2-1. The peptide hormones vary considerably in size, ranging from a few amino acids to full-scale proteins. For example, the hypothalamic factor that induces the release of thyroid-stimulating hormone (TRH or TSH-releasing hormone) consists of only three amino-acid residues, whereas follicle-stimulating hormone (FSH or follitropin) and thyroid-stimulating hormone (TSH or thyrotropin), both released from the anterior pituitary, are heterodimeric glycoproteins (with a common α-chain and a specific β-chain), comprising roughly 245 amino acids each. Thyroid hormones, triidothyronine (T3) and thyroxine (T4), are particular in that they are tyrosine-based hormones that incorporate three and four iodines, respectively.

Growth- and differentiation factors

The discovery of growth factors originates from the finding that cells kept in culture require serum, the soluble component of clotted blot, in order to proliferate. Classic examples are platelet-derived growth factor (PDGF A, B, C), fibroblast growth factor (FGF 1–22), epidermal growth factor (EGF), insulin-like growth factor (IGF 1, 2), vascular endothelial cell growth factor (VEGF A–D), or transforming growth factor-β (TGFβ 1–3). They are of polypeptides with sizes varying from 5.7 kDa for Insulin, 6 kDa for EGF, 25kDA for VEGF, to 78 kDa for Transferrin (a protein also involved in the transport of Fe^{3+} ions). As with the hormones, cells have specific high-affinity-binding sites for growth factors. Apart from stimulating cell proliferation (growth), they also may, depending on context and cell type, inhibit proliferation, initiate programmed cell death (apoptosis), or lead to cell differentiation. The effects of growth factors generally last for days. Many cells produce growth factors, there are no specific glands. There are always exceptions, for instance IGF-1 is mainly produced by the liver in reply to the presence of GH, which is a "growth factor" released into the circulation by the anterior pituitary. Production may be enhanced locally, for instance after wounding.

Certain growth and differentiation factors do not diffuse at all; they remain attached to the membrane and operate in a juxtacrine fashion. Good examples are Wnt (ligand of the Frizzled and LPR receptors) and Delta1 (ligand of the Notch receptor). Wnt and Notch are particularly involved in cell fate decisions, both during the development and in stem cells of adult tissues.

Neurotransmitters

Neurotransmitters came to light in pharmacological studies using various plant alkaloids and animal gland extracts that were known to affect nerve transmission in the peripheral CNS (causing paralysis or muscle contraction, increased vascular tone, etc.). They are stored in vesicles in the presynaptic nerve cell and are released locally, upon an appropriate signal, into the tiny volume defined by the synaptic cleft. The released transmitter then diffuses across the cleft and binds to receptors on the postsynaptic cell (another neuron or effector organ like muscle). On the macroscopic scale, diffusion is a slow process, but across the short distance that separates the pre- and postsynaptic neurons (~0.1 μm or less), it is fast enough to allow rapid communication between nerves or between nerve and muscle at a neuromuscular junction. Table 2-2 shows the structures of some important neurotransmitters. In the CNS glutamate is the major excitatory transmitter (increasing the frequency of action potentials), whereas GABA and glycine are inhibitory (reducing spike frequency). Acetylcholine has its most prominent role at the neuromuscular junction where it is excitatory. Whether an excitatory or inhibitory effect is evoked depends on the nature of the ion channel that is regulated by the neurotransmitter receptor. For a number of neurotransmitters, receptors have been found outside the synaptic cleft, which means that neurotransmitters can also act in a paracrine fashion (Bunin and Wightman, 2000).

Cytokines

Cytokines, from Greek cell movement, have been discovered in the study of soluble factors that control innate and adaptive immunity. They have been classed as lymphokines, IL, and chemokines, originally based on presumed function, named after cells that secrete them or are targeted. The vast majority of cytokines are produced by T-helper cells, which plays a key role in orchestrating the immune response. They affect white blood cells (also known as leukocytes, hence the term interleukins), such as type B and T lymphocytes (hence the term lymphokines), neutrophils, monocytes, and macrophages. Important lymphokines include IL, interferons (IFN), and granulocyte–macrophage colony-stimulating factor (GM-CSF). Chemokines are so named because they direct cell migration; they induce a chemokinetic response of the white blood cells. Two prime examples are the homing of lymphocytes to lymph nodes and the infiltration of leukocytes into wounds. Examples of chemokines are interleukin-8 (IL-8), macrophage chemoattractant protein-1 (MCP-1), monocyte-inhibitory protein-1 (MIP-1), and secondary lymphoid-tissue chemokine (SLC/exodus-3). Tissue necrosis factor-α (TNFα), the growth factor TGFβ, erythropoietin (EPO), and thrombopoietin (TPO) also qualify as cytokines.

Inflammatory mediators and vasoactive agents

Keeping the appropriate blood pressure and facilitating the recruitment of white blood cells to sites of infection/inflammation are two key responsibilities of vasoactive agents. With respect to vascular tone, angiotensin is a very effective mediator. It is derived from a precursor molecule, angiotensinogen, produced by the liver and causes vasoconstriction. It also causes thirst and promotes sodium retention in the kidney through induction of expression of the hormone aldosterone (a mineral corticoid from the adrenal gland). Both mechanisms lead to a prolonged increase in blood pressure. Adrenaline, which normally qualifies as a hormone, also plays an important role in regulating the vascular tone, both by increasing the force and rate of the heart beat and by causing vasoconstriction. Atrial natriuretic peptide (ANP), released from overstretched cardiac atrium muscle cells, does the opposite, it relaxes vascular smooth muscle and reduces salt retention in the kidney, thereby reducing blood volume and thus pressure. Nitric oxide (NO), a gas with a half life of only a few seconds, also plays an essential role in keeping the right vascular tone. It relaxes vascular smooth muscle cells and was long known as "endothelium-derived relaxing factor" (but without a clue about its composition). It is produced by endothelial cells and lives just long enough to attain the smooth-muscle layer surrounding small vessels. Nitric oxide is also generated by neutrophils, monocytes, and macrophages and in this setting it plays a role in the killing of bacteria, particularly those that grow inside macrophages such as *Mycobacterium tuberculosis* and *Salmonella enterica* (Segal, 2005).

At sites of infection/inflammation, the vascular bed increases in volume, a process referred to as vasodilation. The endothelium also becomes more permeable, the junctions between the cells are loosened, and this facilitates exudation of plasma and extravasation of blood-borne cells. Inflammatory mediators that bring this about include histamine (released by mast cells and basophils) and bradykinin (from Greek, slow movements, released by a variety of cells). Serotonin acts in the opposite direction. It is released by platelets upon their clotting as a consequence of endothelial damage and it acts as a vasoconstrictor to stop bleeding. All the aforementioned vasoactive agents are small molecules. Adrenaline is derived from tyrosine, histamine from histidine, and serotonin (or 5-hydroxytryptamine) from tryptophan. Angiotensin, bradykinin, and ANP are short peptide chains, ranging from 9 to 17 amino acids. They are derived from cleavage of large precursor proteins, angiotensinogen, kininogen, and preproANP, respectively.

The eicosanoids are another important family of vasoactive compounds. They are derived from the three possible sources of polyunsaturated fatty acids: arachidonic acid (ω-6), dihomo-γ-linolenic acid (ω-6), and eicosapentaenoic acid (ω-3). They are termed eicosanoids (from the Greek word for 20) because they originate from a 20 carbon fatty-acid chain. They include the prostaglandins (vasodilator), thromboxanes (clotting factor and vasoconstrictor), and leukotrienes (vascular permeability and chemotaxis) and they operate over short distances in a paracrine or autocrine fashion, controlling many physiological and pathological cell functions. In the context of inflammation, they cause plasma leakage, skin reddening, and the sensation of pain. Together with histamine and bradykinin, they are responsible for the four signs of inflammation: redness (rubor), swelling (tumor), heat (calor), and pain (dolor). The drugs aspirin (acetylsalicylic acid) and paracetamol (members of the nonsteroidal anti-inflammatory drugs) possesses anti-inflammatory, anti-coagulant, and pain-relieving properties because they inhibit cyclo-oxygenase (COX), a key enzyme in the pathway generating prostaglandins and thromboxanes, such as the vasodilator and hyperalgesic prostaglandin E2 and the thrombotic TXA2.

Other, nonclassical, members of the eicosanoids are lipoxins, cannabinoids (Figure 2-3), lysophosphatidic acid, and platelet-activating factor.

OMEGA-3 AND -6 (ω-3 AND ω-6)

Of the possible sources of eicosanoids, two are omega-6 fatty acids, arachidonic acid and dihomo-γ-linolenic acid, whereas one is an omega-3, namely eicosapentaenoic acid. Omega (ω) refers to the position of the double bond at the tail of the fatty acid (see Figure 2-3 for arachidonic acid). The metabolites emanating from these three sources are not the same. This difference lies at the origin of the debate about the role of

continued

OMEGA-3 AND -6 (ω-3 AND ω-6) *(cont'd)*

dietary fatty acids in public health. The current paradigm states that eicosanoids derived from ω-6 arachidonic acid generally promote inflammation, whereas those derived from ω-3 eicosapentaenoic acid and from ω-6 dihomo-y-linolenic acid are generally less active or even involved in the resolution of inflammation.

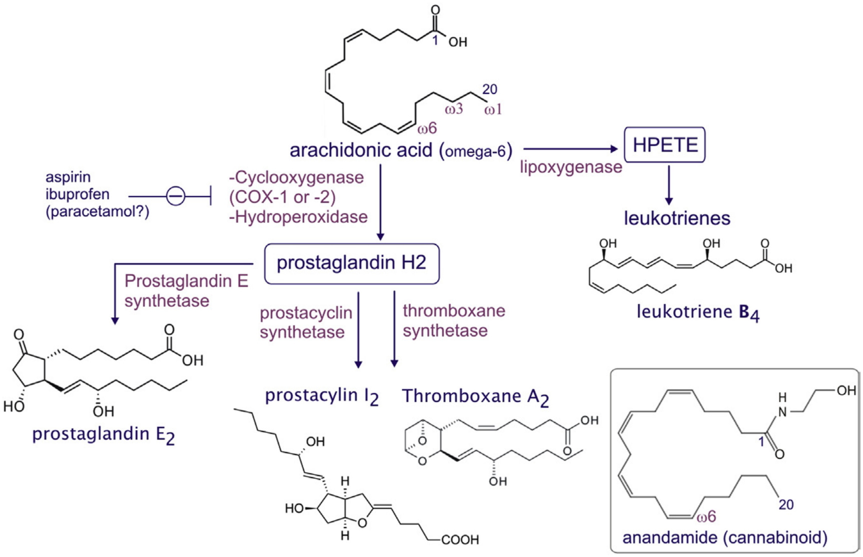


FIGURE 2-3 Examples of eicosanoids.

Hydrophobic messengers

There is a class of hormones that differs from the first messengers that we have examined so far, these are the steroid hormones. They are synthesized from cholesterol, by the mitochondria and the smooth endoplasmic reticulum, in the gonads and the cortex of the adrenal gland. They are hydrophobic in nature and require lipid carriers for transport in the blood. They insert into the plasma membrane and are then recognized by intracellular receptors (or intracellular carrier proteins) that translocate the hormones to the nucleus, where they modify gene expression. They are classified into five groups according to the receptor to which they bind: glucocorticoids (glucose metabolism and inhibitor of inflammation), mineralocorticoids (sodium retention in kidney), androgens (male development), estrogens (female development), and progestogens (pro-gestational, maintaining pregnancy).

The extracellular matrix and neighboring cells

Apart from soluble mediators, both the extracellular matrix and neighboring cells act as (solid) first messengers. Matrix components such as fibronectin, laminin, glycosaminoglycans, or collagens interact with cell adhesion molecules, members of the integrin and the "link" family of adhesion molecules. Adhesion molecules certainly play a role in physical adherence to the extracellular matrix but they also qualify as genuine receptors, we speak of outside-in signaling. Signaling also occurs in cellular junctions, for instance through the contact between cadherins in the desmosome or in the adherens junction of epithelial cells.

FIRST-MESSENGER SIGNALS ARE AMBIGUOUS: THEIR MEANING IS EMBEDDED IN CONTEXT

The ultimate effects of these first messengers are determined, not by their chemical natures, but by the anatomical context within which they operate (type of cell and its environment) and the characteristics of the receptors that sense their presence (Figure 2-4). This explains why the

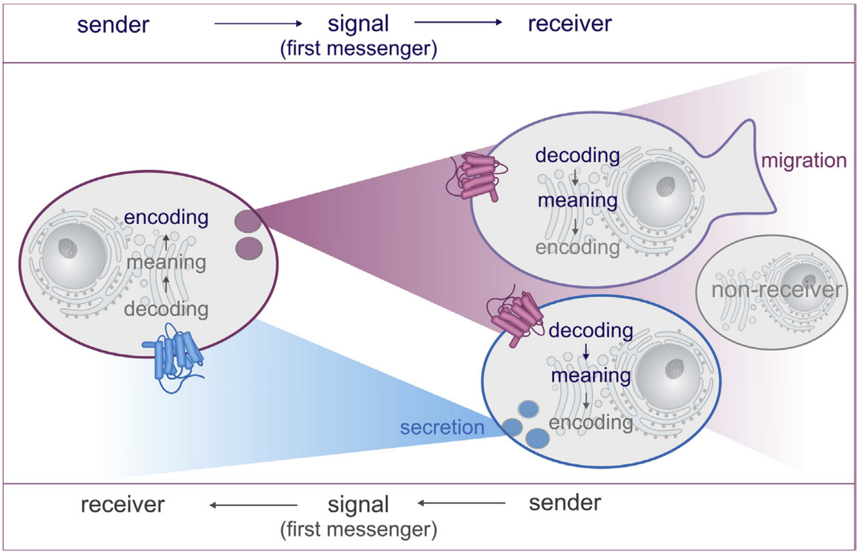


FIGURE 2-4 The signal (first messenger) connecting the sender with the receiver is ambiguous; it carries no intrinsic message. The response to the signal depends on the presence of a receptor and on the cellular context; one type of cell replies to the same first messenger with migration, the other with secretion. Cells that lack the appropriate receptor do not respond. If the response of the receiver (blue cell) constitutes the secretion of a first messenger then the receiving cell turns into a sender and may affect the cell it received the signal from in the first place. There is no real hierarchy in cell communication; organisms function in a bottom-up approach.

same messengers are used in many different situations, ranging from roles as diverse as early embryonic development to maintenance and repair in adult organisms. Molecular analysis has revealed that the same messengers are used across many different species, ranging from prokaryotes to complex multicellular organisms. In the light of evolution, it is postulated that diversity of organisms occurs through the employment of core components (comprising first messengers, their receptors, and effectors) that operate in new amounts and states at new times and new anatomical locations (Gerhart and Kirschner, 2007).

TANGLED HIERARCHY OR STRANGE LOOP

We have deliberately created a loop in Figure 2-4 with the aim to illustrate that senders and receivers are not really separate anatomical entities. It is not an essential message for understanding signaling pathways but it is an important message for understanding the full interdependence of all processes in living organisms. In the chapter about Notch we discuss a very clear example of how two adjacent cells determine each other's destination (cell fate). To illustrate this interdependence further we refer to one of the works of the Dutch artist Maurits Escher because his lithograph, "tekende handen" (drawing hands) is remarkable in this respect (Figure 2-5). The hand that draws has arisen because it is being drawn by the other hand. The situation where there is an apparent but not a real hierarchy is referred to as "tangled hierarchy" or "strange loop" (Hofstadter, 1979). Moreover, the lithograph manifests a paradox: if you would trace back to the origin, how did the process get started? This, according to Hofstader, represents another "strange loop"; the emergence of life out of lifeless material.

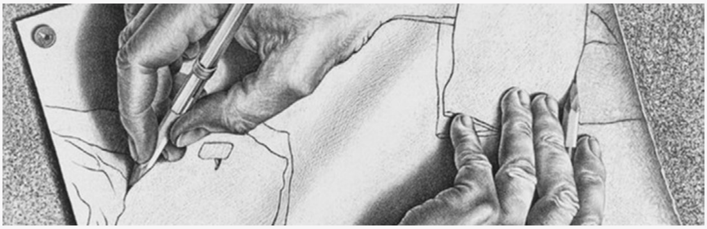


FIGURE 2-5 Drawing hands, a lithograph depicting interdependence. *Drawing from Escher, M.E., 1948. Original drawings can be seen at http://www.mcescher.nl/.*

THE PLASMA MEMBRANE BARRIER, MEMBRANE RECEPTORS, AND SIGNAL TRANSDUCTION

In the main, when we consider signal transduction we are concerned about how first messengers can determine what happens on the inside of their target cells. There is a difficulty, since nearly all the first messengers are hydrophilic and thus unable to pass through membranes (the long fatty-acid chains do not offer any electrostatic interactions that satisfy hydrophilic substances). Their influence must somehow be exerted from outside. To remind you, the membranes of cells, although very thin (4–6 nm) are effectively impermeant to ions and polar molecules. To illustrate this, while K^+ ions might achieve diffusional equilibrium over this distance in water in about 5 ms, they would take some 12 days to equilibrate across a phospholipid bilayer (under similar conditions of temperature, etc.). So, for a hormone such as adrenaline the rate of penetration into a cell is too low to measure. The evolution of membrane receptors has accompanied the development of mechanisms that permit external signaling molecules, the first messengers, to direct the activities of cells in a variety of ways with high specificity and precise control in terms of extent and duration. With some important exceptions (the steroid- and thyroid hormones and nitric oxide), they do this without ever needing to penetrate their target cells. In first instance, the signal, not the messenger, is carried into the cell through the receptor and this process is referred to as signal transduction. Transduction refers to the combined process of *bringing across* and *translating* (decoding) the extracellular signal. Signal transduction thus comprises all processes whereby engaged receptors (both plasma membrane and intracellular) transmit signals into the cell. Collectively, these provide a symbolic representation of the environment and allow cells to respond appropriately to changes (both early and late responses) (Figure 2-6).

It is important to notice that receptors too are internalized, through the process of endocytosis, in particular when bound to first messengers. Evidence has been provided that internalized receptors continue to signal for some time. However, the first messengers never enter the cytosol; they remain inside endosomes and are, after separation from the receptor, destroyed in lysosomes. In many instances, the receptor is recycled and reappears at the membrane.

RECEPTORS AND THEIR LIGANDS

From here on we will replace the term "first messenger" with that of "ligand." The reason we do this is because in the numerous studies dealing with how substances interact with receptors, that is, measurements of affinity, conformational changes of the receptor, and so on, the term ligand

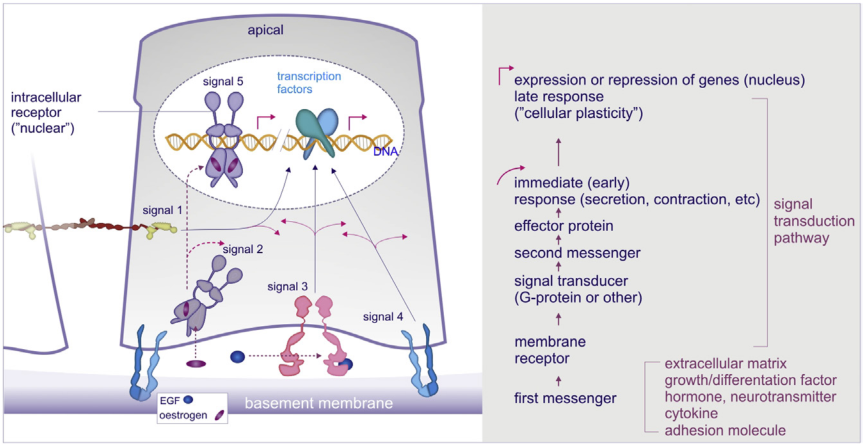


FIGURE 2-6 The process of signal transduction. Multiple receptors receive (different) signals from "soluble" and "solid" factors. Collectively these provide a symbolic representation of the cellular environment, both near and remote.

is most common. A ligand is defined as a molecule that binds to a receptor in a specific and saturable manner. The term ligand refers to a wide range of endogenous (produced by and operating in the organism in question) and exogenous substances (produced by plants, fungi, bacteria, or chemists and applied to another organism). In the language of pharmacology, binding of a first messenger to its receptor is termed "ligand binding" or "receptor occupation." Binding of the ligand to its receptor is in nearly all cases non-covalent. An exception is the light-sensitive chromophore retinal, covalently bound to rhodopsin, the light receptor in the retina. For small ligands, such as hormones and neurotransmitters, the interaction with the receptors occurs through electrostatic bonds (long and short distance, attractive or repulsive) as well as "van der Waals" bonds (short distance, always attractive). For large ligands, hydrophobic attraction between extensive surfaces of the ligand and receptor may also play an important role. An example of a topographically localized (adrenaline) and topographically extended (EGF) interaction is shown in Figure 2-7. The interactions are reversible; the ligand binds to its receptor and then, after some time, separates from it. In the case of growth factors like EGF, binding is so strong that dissociation only occurs under acidic conditions in the endosome. On average, it is estimated that cells express roughly 10,000 copies per type of receptor. Below we will discuss the importance of receptor numbers in making the system sensitive.

LIGAND

The term "ligand" was first proposed by chemists to describe an electron donor group forming coordination complexes with metal ions, that is, sites which secure binding of Fe^{2+}, Mn^{2+}, and other metals within macromolecules such as NADH, cytochrome *c*, or phosphatases.

LIGAND AND RECEPTOR TERMINOLOGY BEYOND SIGNAL TRANSDUCTION

The term ligand and receptor are not limited to signal transduction. The same terminology is used to describe mechanisms involved in the routing of protein, where specific receptors recognize short amino acid sequences in cellular proteins. For instance, the signal peptide, a twenty-or-so N-terminal sequence present in proteins that are destined for the rough endoplasmic reticulum, binds the Signal Recognition Particle in a ligand/receptor fashion. Proteins destined for mitochondria bind to specific receptors in the outer membrane, termed Tom70 and Tom20, and proteins that enter a peroxysome must first be recognized by the cytoplasmic receptor PEX5. In short, the term ligand and receptor apply very broadly but always refer to a precise nonenzymatic (a condition where we employ the term "substrate") interaction between two molecules.

SOME FIGURES TO ILLUSTRATE THE SIZE OF THE ISSUE

There are many receptors and they nearly all are protein in nature. Introduction of "*Homo sapiens* receptor" in the "Entrez Gene" database (National Institute of Biotechnology Information (NBCI)) results in a list of 5164 genes that encode receptors (identified by their sequence patterns). The same operation in the "UniProtKB/Swiss-Prot" data bank (ExPASy) provides a list of 1418 cellular proteins, which are identified as receptors or components of a receptor complex. From this it follows that a large number of receptors remains to be functionally characterized. The first messengers also are abundant but, as we have shown, they are not always of a protein nature and therefore cannot be explored by the above-mentioned databases. First messengers may have several different receptors, so that receptor numbers exceed those of first messengers. A case in point is serotonin (5-HT) for which to date 13 receptors have been identified. We now recognize about 200 proteins as growth factors, roughly 100 molecules have the status of neurotransmitter (or neuromodulator) and dozens of molecules qualify as hormones or cytokines.

Receptor-binding characteristics

When observing a single ligand over a given period of time (a few nanoseconds) and measuring the duration of binding to its receptor, we define a low-affinity ligand as one that spends most of the time

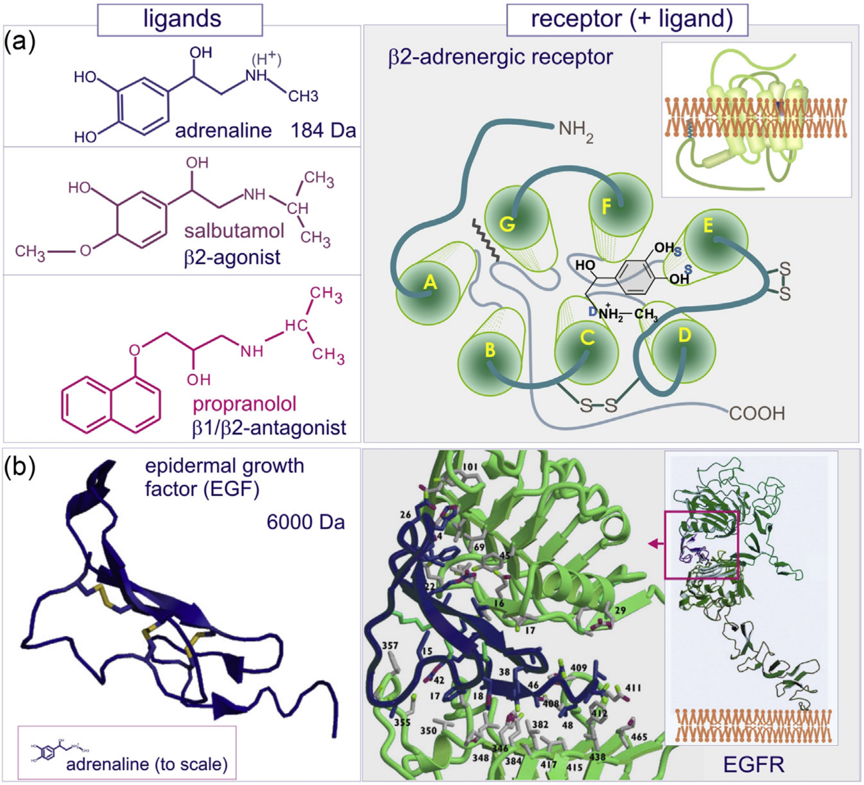


FIGURE 2-7 An example of a low and a high molecular mass ligand bound to its receptor. Note the topographically localized interaction between adrenaline and its receptor and the numerous, extended, interactions that occur in the case of EGF.

detached. A high-affinity ligand, on the contrary, will spend most of that time attached to its receptor. In order for the receptor to be occupied for some time, low-affinity ligands must constantly replace one another; one ligand leaves the receptor, another takes its place. In other words the ligand must be present in high concentrations, in the micromolar (μM) range. High-affinity ligands can keep their receptors occupied at low concentrations, in the nanomolar (nM) range (Figure 2-8). The advantage of high-affinity ligands is their efficacy; you need few molecules to occupy a good number of receptors and trigger an intracellular signal. The advantage of low-affinity ligands is their reversibility; removal instantly results in a drop of occupied receptors. For rapid on/off responses, like muscle contraction, low affinity is to be favored. When restricting the diffusion area, like in a synaptic cleft, high concentrations are easily achieved, without having to produce large quantities.

NUMBERS

A hormone may elicit physiological effects at concentrations as low as 10^{-10} mol/l ($10^{-10} \times 6 \times 10^{23} = 6 \times 10^{13}$ molecules per liter). To get an idea of how many of these molecules an individual target cell might encounter, consider that a 12 µm diameter cell occupies a volume of 10^{-12} l. This volume of extracellular fluid would contain only 60 molecules of hormone. In spite of the necessary high affinity, it is important to remember that the binding interaction is never covalent and is always reversible.

The value of 60 molecules is minute when comparing it with the number of ions of electrolyte present in the same volume - $\sim 1.8 \times 10^{11}$ (for isotonic saline). Furthermore, the same volume of intracellular water will contain about 10^{10} molecules of ATP (between 5 and 10 mmol/l) and after hormonal stimulation, a cell might contain as many as 10^{6} molecules of the second-messenger cAMP (second messenger produced after receptor occupation).

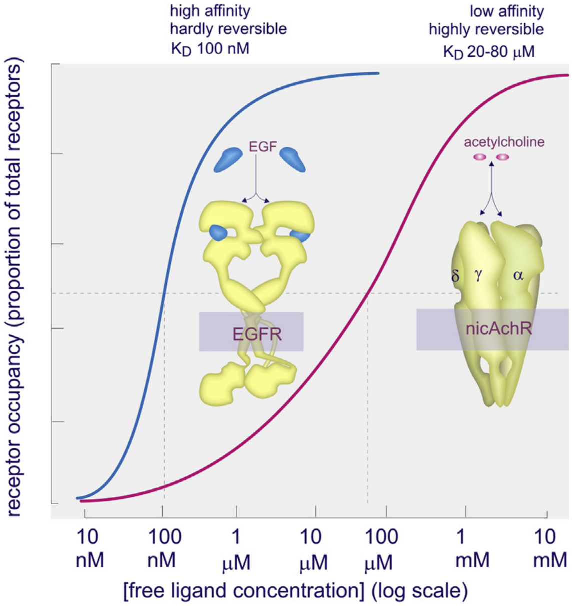


FIGURE 2-8 Two different approaches to receptor-ligand interaction: fast, reversible, and wasteful versus slow, nearly irreversible, and economical.

Receptors are not solid structures, carved out of marble, with one precise binding pocket for an appropriate ligand. We return to this subject in many instances but receptors are proteins and proteins are flexible. The current understanding is that receptors oscillate between different conformations.

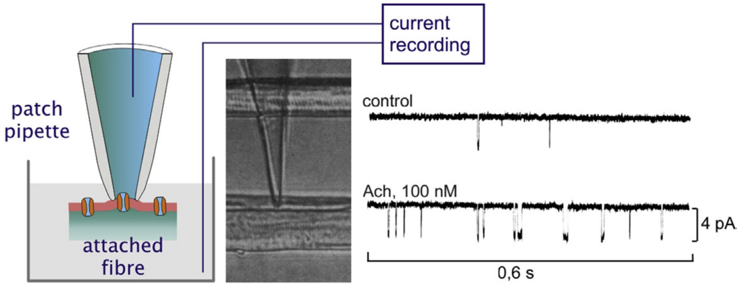


FIGURE 2-9 Open and close probability of the nicotinic acetylcholine receptor in the absence (control) and presence of acetylcholine (Ach, 100 nM).

Some of these conformational states correspond to their "active state." Although the changes are slim, receptors may reach the active state spontaneously and induce signaling events without a ligand. In the context of signal transduction we define the "active state" as the state that either leads to interaction with other cellular components (binding to DNA or to G-proteins), increased enzymatic activity, or opening of ion channels. Indeed, it is well recognized that ligand-gated ion channels exist in discrete states, typically "open" or "closed." Under resting conditions ligand-gated ion-channels are mainly closed but from time to time they do open spontaneously. The open-state probability increases enormously in the presence of ligand (for instance, acetylcholine) (Figure 2-9). Receptor activity in the absence of ligand has also been clearly demonstrated in Sf9 insect cells that were forced to express the β2-adrenergic receptor. These cells, normally devoid of such receptors, started to produce second messenger in proportion to the total number of expressed receptors and, importantly, in the total absence of adrenaline (Chidiac et al., 1994). Receptors thus exist in different functional states, with relatively low energy barriers to move from one to another (Deupi and Kobilka, 2010). In electronic engineering, unsolicited or undesired signals are called "noise." Accurate measurements, like sensing the cellular environment for the presence of first messengers, require low noise and strong signals (or a high signal-to-noise ratio).

The current understanding for β-adrenergic receptors, one of the best studied receptors, is that ligands that activate (i.e., give rise to signaling events), increase the free-energy state of the inactive conformation and reduce the free energy of the active conformation and hence increase the proportion of active receptors. In pharmacological speak, activating ligands are called "agonists." Ligands that increase the proportion of inactive receptors, by augmenting the free-energy state of the active conformation, are called "inverse agonists." "Antagonists" distinguish themselves by not pushing receptors in either direction; their role is to block access of agonists (or inverse agonists) (Figure 2-10). The situation of free-energy states is influenced by

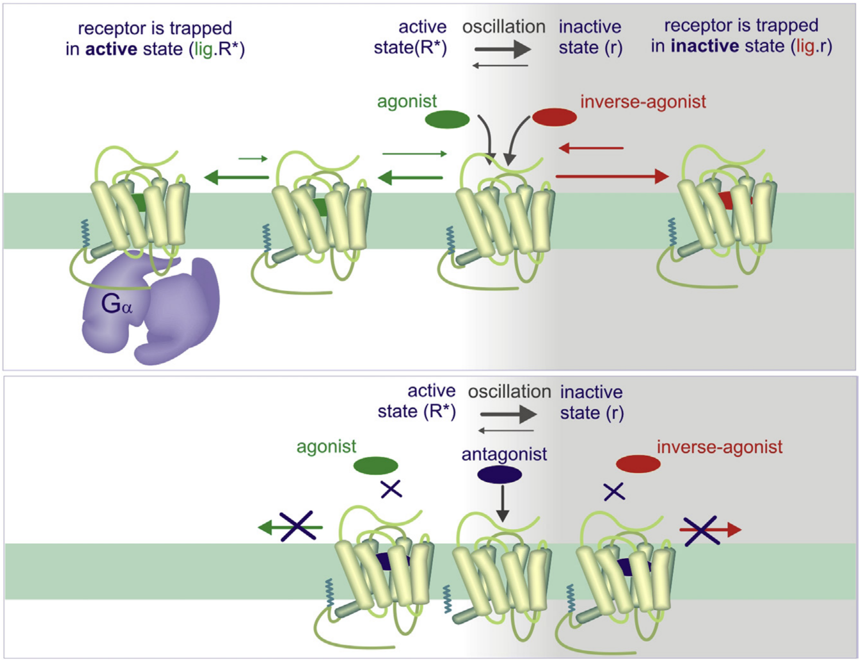


FIGURE 2-10 Mode of action of agonist, antagonist, and inverse-agonist. Agonists lower the free-energy state of the active receptor (going to the left). Subsequent interaction with G-proteins lowers the energy level even further and the ligand/receptor/G-protein complex is trapped in an active state. Inverse agonists do the reverse; they reduce the free-energy state of the inactive receptor (which cannot interact with G-proteins). Antagonist do not affect free-energy states, they simply block access of agonists (and inverse agonists) and thus prevent signaling.

the interaction of the occupied receptor with downstream signaling components (in the inside of the cell). The occupied β2-adrenergic receptor interacts with GTP-binding proteins (G-proteins), and this interaction further diminishes the free energy of the receptor–agonist complex thereby preventing the spontaneous relaxation of active conformation to an inactive-like conformation; the complex is said to be trapped in the active state (Rosenbaum et al., 2011). The phenomenon where ligand, receptor, and an associated protein affect each other's shapes in order to arrive to a minimal energy state is called "induced fit." We must add here that posttranslational modifications of receptors, like covalently bound phosphate will also influence the energy states and thus affect the conformational states of receptors. Numerous receptors in their active state occur as dimeric or trimeric complexes. For certain receptors the ligand acts as a bridging molecule, in other receptors they bring about molecular changes that lead to receptor dimer- or trimerization, in yet other cases the receptor is permanently coupled to other receptors (Kenakin and Miller, 2010). The complexity of receptor activation processes must reflect the need to keep spontaneous signaling (noise) to a minimum.

SHAPE SHIFTING AT BODY TEMPERATURE

Proteins may oscillate between different shapes, not all having the same level of free energy. At 310 K (36.8 °C), shape changes that require not more than 2.3 kcal occur rapidly (in nanoseconds) but larger changes, exceeding a shift of 2.3 kcal may take milliseconds.

We have learned that ligand concentration and affinity for its receptor are important aspects of signal strength, the other is receptor numbers. With a given ligand and concentration, the number of occupied receptors (in an equilibrium situation) is proportional to the total number of receptors expressed on the cell surface. In other words, cells that express high copy numbers of receptors are more sensitive than cells that express low copy numbers (see Figure 2-11). This is why pharmacologists, when employing radioactive ligands, repeatedly found that the number of occupied receptors needed for a maximal cellular response is but a fraction of the total number of receptors present on the cell. The maximum number of receptors is measured by progressively increasing the radioactive ligand until saturation is achieved. At saturation binding all the available receptors are occupied (these studies are performed at low temperature to

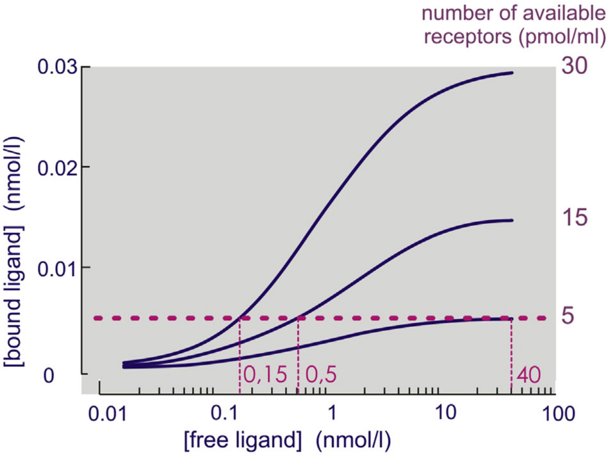


FIGURE 2-11 The percentage of occupied receptors, at a constant concentration of ligand, is determined by the total number of receptors expressed on the cell surface. High receptor numbers make the system more sensitive and that is why dogs smell so much better and certain tumors respond excessively to growth factors. Note that by raising receptor numbers from five to 30 (pmol/ml), you require 266 times less ligand in order to achieve the same number of occupied receptors.

prevent receptor uptake). Quite erroneously, these unoccupied receptors were labeled as “spare receptors” (which they are certainly not). Cells have ways to regulate the number of receptor-copies in their plasma membrane (often referred to as “regulation of cell surface expression”). For instance, when confronted with a sustained high concentration of a first messenger, cells tend to reduce its impact by removing receptors from their surface. This is a feedback phenomenon referred to as “receptor down-regulation.” Cells have other ways to diminish the impact of first messengers, namely by rendering the receptors refractory. This mode of desensitization can occur through phosphorylation of the intracellular segment. In this situation, receptor numbers remain the same but the response is blocked. Disease may occur as a consequence of the inappropriate cell surface expression. Excessive receptor expression occurs in certain types of breast cancer. In this particular setting, duct epithelial cells of the mammary gland have amplified the gene coding for ERBB2 (located on chromosome 17), which codes for one of the four different types of EGF receptors. The type-2 EGF receptor is massively expressed and renders the epithelial cells very sensitive to EGF (besides an increased level of unsolicited signaling). This sensitivity contributes to the process of cell transformation (a process where cells lose their polarity, increase their proliferation, and disseminate into the organisms (metastasis)). An example where the loss of receptors causes disease is Myasthenia Gravis. The symptom is muscle weakness (Greek: myo, muscle; asthenos, without force) and this is caused by a loss or a dysfunctioning of the nicotinic acetylcholine receptor due to autoimmunity (antibodies raised against self). The low number of (effective) receptors in the neuromuscular junction often fails to generate action potentials and, as a consequence, very few motor units contribute to muscle contraction. Both boosting the concentration of acetylcholine in the synaptic cleft, by a cholinesterase inhibitor such as neostigmine, and inhibiting the immune system, by a variety of means, generally improves muscle strength to satisfactory levels. In a pharmacological perspective, effective receptor numbers can also be reduced by simply blocking the access of ligands by the provision of antagonists. They are widely used in the treatment of disease. The β-adrenergic receptor antagonists, such as propranolol or timolol, are prime examples. They are known as “beta-blockers” and are very effective in the treatment of angina pectoris (stable angina) and cardiac arrhythmias.

A last point to remember is that the relation between receptor occupation and cellular response is hardly ever linear (Stephenson, 1956). We just mentioned that in many instances full cellular responses were obtained with a small fraction of the receptors being occupied. In order to describe the relationship between ligand concentration and cellular responses, the term EC50 has been introduced. This value expresses the effective concentration of a ligand needed to induce a half-maximal

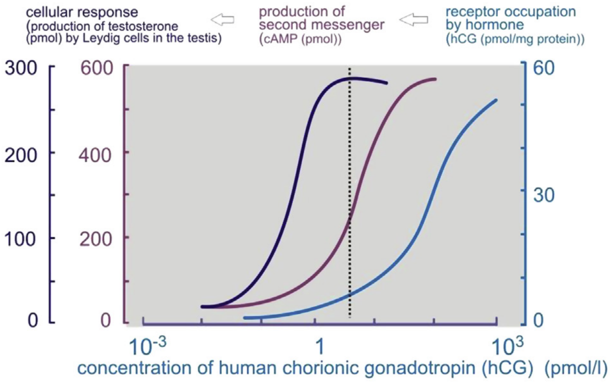


FIGURE 2-12 Relationship between receptor occupation (hCG binding), signaling activity (production of cAMP), and cellular response (production of testosterone). Going from right to left: a tiny proportion of occupied receptors suffices to generate a nearly half maximal signaling activity which subsequently gives rise to a full cellular response. The EC50 for receptor occupation is around 100 pmol/l, for second-messenger production 6 pmol/l, and for the cellular response 0.6 pmol/l.

response (50%) in any given tissue or cell population. The EC50 value naturally depends on what we actually measure. We can measure muscle contraction, saliva secretion, or cell division but we can also study precise intracellular events, like changes in metabolic pathways or events occurring in the signal transduction pathway downstream of the receptor. Figure 2-12 illustrates the relationship between receptor occupation and cellular responses measured at two levels: one, the production of a second messenger (signal transduction) and two, secretion of testosterone. The EC50 value can be shifted by manipulations that affect ligand binding (addition of antagonists) but also by the treatment of cells with agents (drugs) that alter the activity of the enzymes that take part in the events that follow receptor activation.

Five types of receptors

For pedagogical purposes we recognize five types of receptors based on their structure or signaling pathways they employ. They are: (1) the ligand-gated ion channels; (2) seven-membrane-spanning proteins coupled to GTP-binding proteins (the GPCRs); (3) receptors with a single membrane pass and which carry an enzymatic activity in the cytosolic segment; (4) receptors with a single membrane pass and which couple to protein kinases; and (5) nuclear hormone receptors (Figure 2-13). As with all classifications, some receptors do not fit any of these global categories.

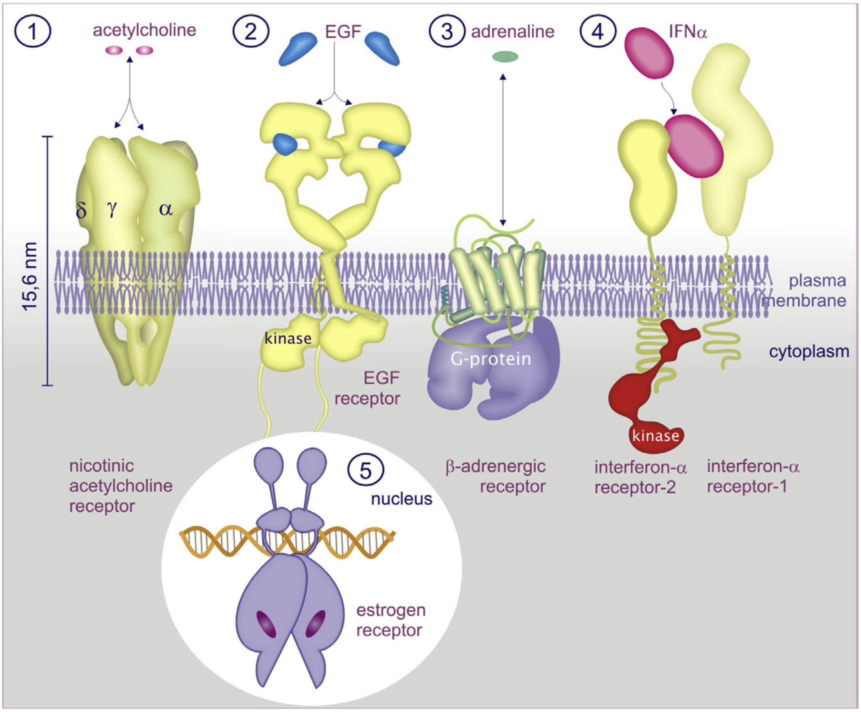


FIGURE 2-13 Five types of cell receptors involved in signal transduction. PDB files used for representation of the receptor: (1) nicAchR, 2bg9, (2) EGFR, 1ivo + 2gs6, (3) bAR, 2rh1, (4) IFNAR2/IFNa, 2kz1, and (5) ER, 1a52 + 1hcq. Objects are more or less to scale.

Type-1 receptors are ligand-gated ion channels involved in the rapid transmission of signals across the synapse between two electrically excitable cells. This type of transmission occurs via neurotransmitters such as ATP, glutamate, glycine, acetylcholine, or serotonin. They have varying specificity for ions, they all open transiently upon binding of the ligand. For certain channels, regulation of ion-transport occurs through the membrane potential, they are the so-called voltage-dependent ion channels. Examples of ligand-gated ion channels are shown in Figure 2-A.

Type-2 receptors are G-protein-coupled receptors (GPCR) characterized by a common seven α-helical transmembrane architecture (7TM) and, as their name infers, by signaling through heterotrimeric GTP-binding proteins (G-proteins composed of α-, β-, and γ-subunit). GPCRs form the largest family of membrane receptors, approximately 800 different receptors in the human genome, and they are all related to rhodopsin, the first GPCR discovered in the rod outer segments of photoreceptor cells in the retina. The ligands of this receptor type are extremely varied. They range from retinal activated by a photon (for rhodopsin), odor molecules, lipids (e.g., prostaglandins), to relatively large polypeptide hormones (LH, FSH, etc.), and growth factors (Wnt, thrombin). Olfaction alone involves about 400 different receptors (but we note that the numbers vary significantly per year and per information source). Based on their amino-acid sequence, GPCRs are classified into five subfamilies: rhodopsin, secretin, glutamate, adhesion, and frizzled/taste-2 receptors (Fredriksson et al., 2003). GPCRs are frequent targets of therapeutic drugs. However, the function of many GPCRs, as well as the identity of their ligands remains to be established (there are still many "orphan receptors"). Examples are shown in Figure 2-B.

Type-3 receptors represent receptors that have a single membrane pass and carry an enzymatic activity in their intracellular segment. With one exception, this is a tyrosine or serine/threonine kinase activity. These receptors form dimers after ligand binding (with the exception of the insulin receptor that is already dimerized) and the majority undergoes phosphorylation of their cytoplasmic segment in order to render the kinase domain catalytically competent (activation process). Ligands of this receptor type belong mostly to the family of growth factors such as EGF, FGF, PDGF, insulin, and M-CSF among others. We have grouped the receptor for atrial-natriuretic peptides (ANF and ANP) among type-3 because they are single-pass membrane proteins with a serine/threonine kinase. However, because of an inactivating mutation, their kinase domain is dead (pseudo-kinase) and these receptors signal with their guanylyl cyclase activity (production of the second-messenger cGMP) (Lowe et al., 1989). Examples are shown in Figure 2-C.

Type-4 receptors represent receptors that have a single membrane pass but lack an intrinsic kinase domain. In order to signal, these receptors associate with protein kinases located in the cytoplasm. Association can be direct, like for instance the interferon receptor (IFNR) that binds the tyrosine kinases JAK1 and TYK2. In the case of the tissue necrosis factor receptor (TNFR) or the toll-like receptor (TLR), recruitment of serine/threonine kinases occurs through a series of adaptor proteins. The kinases play an important role in transmitting the signal into the cell, much in the same way as described above for type-2 receptors. Examples are shown in Figure 2-D.

Type-5 receptors represent the nuclear receptors. Their ligands are either hydrophobic, traverse the plasma membrane, or they enter the cell through a transporter protein (case of thyroid hormones). They are so named because receptor–ligand complexes accumulate in the nucleus. Indeed these receptors are DNA-binding proteins and they function as transcription factors but a good deal of them has membrane-localized actions as well. In this group we include retinoid receptors (RXR and RAR), steroid receptors (androgen AR, estrogen ER, progesterone PR, glucocorticoids GR, and mineralocorticoids MR), thyroid hormone receptors (TR), fatty acids, and intracellular eicosanoid receptors (PPAR). Examples are shown in Figure 2-E.

Miscellaneous receptors

There are receptors that belong to none of the above-described types. The nitric oxide receptor, which is responsible for relaxation of vascular smooth muscle, is one of these. Nitric oxide penetrates the membrane and binds and activates a cytosolic guanylyl cyclase (production of cGMP). The differentiation factor Notch, already described above, is arranged in this miscellaneous group. Adhesion molecules have important signaling activities and the integrins in particular can be considered as ligand-activated receptors. They recruit tyrosine kinases to the membrane and contribute to numerous signaling events when engaged with extracellular matrix (fibronectin, laminin, or other) or adhesion molecules from opposite cells (ICAM, VCAM, or JAM). Other adhesion molecules, like selectins, cadherins, and occludines also play a role in cell signaling events. Examples are shown in Figure 2-F.

Web resources

HUGO gene nomenclature GPCR http://www.genenames.org/genefamilies/GPCR.

SIGNALING MECHANISMS

Receptors, transducers, effectors, second messengers

Here we have arrived at an important point; occupied receptors must emit a signal that brings about changes in cellular function. For instance, they induce secretion of hormones, cause muscle contraction, increase the liberation of glucose from glycogen stores, stimulate antibody production, or cause cell division. In other words, the signal has to perturb cellular homeostasis. The signal emanating from the receptor should reach enzymes involved in metabolic processes, or reach proteins involved in membrane fusion. They must reach the actin/myosin contraction machinery or transcription factors involved in expression of immunoglobulins or enzymes that drive DNA replication. Immediate changes are brought about by modifying protein activity or by changing their subcellular localization and deprive (or provide) them of substrate, provide them with protein partners, remove (or attach) them from DNA or RNA or membrane lipids. There are numerous ways to bring about changes in protein activity or in their subcellular localization and there is no real point in trying to classify the mechanisms because there seem to be more exceptions than rules. It is best to learn them through precise examples. We return to this subject in the section about "Posttranslational modifications involved in signaling events" (page 94).

For the majority of receptors, ligand binding leads to the production of an intracellular messenger, which we call the second messenger. Second messengers are often at the start of a cascade of further events, which collectively we name a signaling pathway. Transducers are defined as the proteins that connect receptors with the enzymes responsible for the production of second messengers. From a historical perspective, the term transducer refers to a heterotrimeric GTP-binding protein (G-protein) that stands in between a seven-transmembrane-spanning receptor (β-adrenergic receptor as an example) and an effector (adenylyl cyclase as an example) (Gilman, 1987) and see Chapter 1, "Prologue". Figure 2-14 illustrates two examples of receptor events leading to the production of second messenger. We have chosen these examples not only to illustrate two radically different molecular approaches but also to show the difference in timescale. The generation of an intracellular Ca^{2+} signal is hundred times faster than the production of cAMP. In a rather free interpretation of the terms "transducer" and "effector," the voltage gated Na^+ channel (NaV1.4) acts as a transducer and the CaV1.1/RyR1 complex constitutes the effector for the nicotinic acetylcholine receptor.

Second-messenger production has not been detected for all signaling pathways, in certain cases the signal is immediately transferred to a transcription factor (case of Notch). This does not necessarily mean that they are not produced; they may simply not have been detected or studied yet. The scheme below depicts a variety of signaling mechanisms. Details will be provided in the following chapters (Figure 2-15).

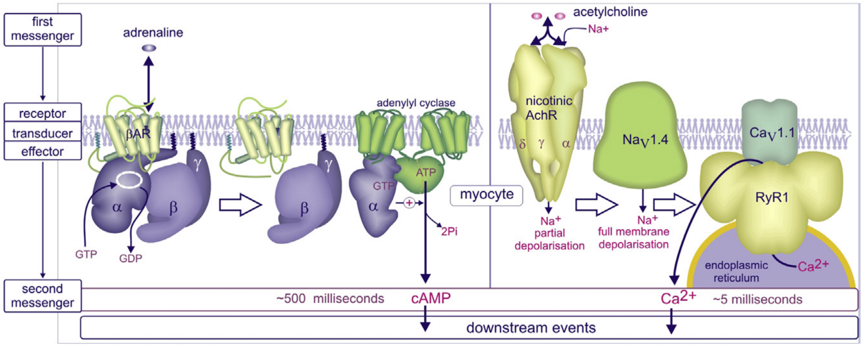


FIGURE 2-14 Two different mechanisms of production of second messengers. Binding of adrenaline (first messenger) to the β-adrenergic receptor leads to an exchange of GTP for GDP in the α-subunit of a G-protein (transducer), which causes dissociation of the complex upon which the Gα.GTP binds adenylyl cyclase (effector). This leads to the production of the second-messenger cAMP. In the case of the nicotinic acetylcholine receptor, binding of the ligand causes an initial inward flux of Na^+, thereby changing membrane polarity and opening voltage-gated Na^+ channels (transducer). In the case of striated muscles this causes the entry of the Ca^{2+} (second messenger) through a channel complex comprising $Ca_V1.1$ and RyR1 (effector). Note the 100-fold difference in time scale between the two mechanisms of transduction.

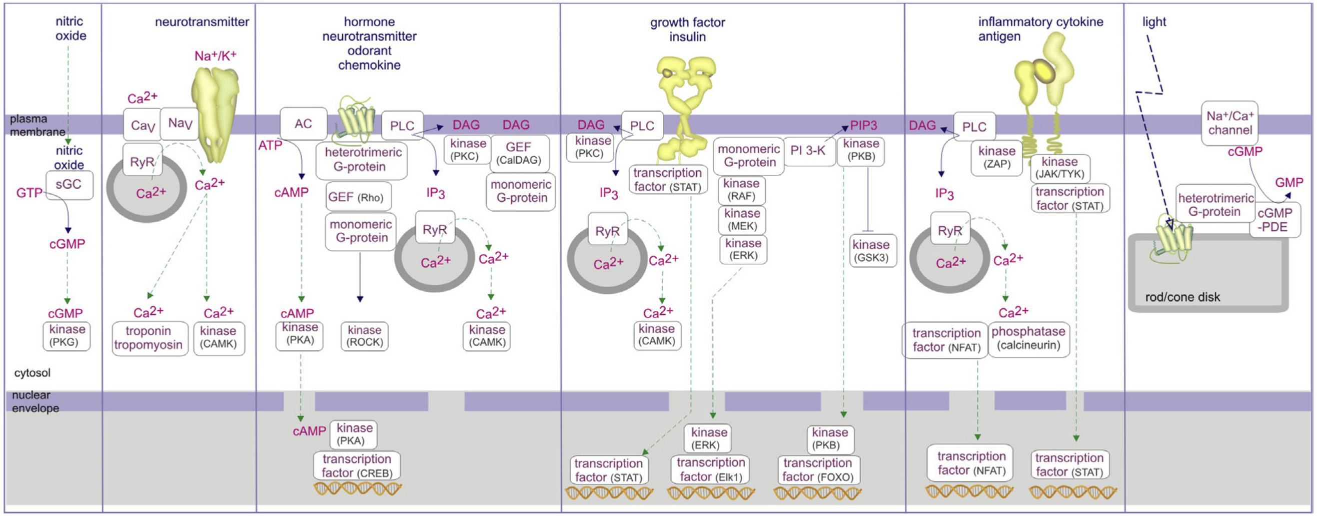


FIGURE 2-15 Schematic overview of a variety of signaling mechanisms.

Putting the components together: the adrenaline to glycogen phosphorylase-signaling pathway

Much of what we know today about signal transduction has been instigated by studies on the effect of adrenaline (and glucagon) on glycogen breakdown in liver or skeletal muscle. With respect to muscle, the context is hard labor. This requires a highly elevated production of ATP in order to keep the myosin heads moving. Sustained ATP production relies on a good supply of glucose-6-phosphate (or alternatively fatty acids) and this is produced through breakdown of muscle glycogen into glucose-1-phosphate (glycogenolysis). Glucose-1-phosphate is converted into glucose-6-phosphate that enters the glycolytic cycle. The resulting acetyl-CoA is next shuttled into the citric acid cycle in order to feed the mitochondrial respiratory chain with electrons (carried by NADH2 or FADH2). The ensuing metabolic process of oxidative phosphorylation leads to the production of ATP. The enzyme that mediates glycogenolysis is glycogen phosphorylase. In cellular homeostasis its activity is controlled by: (1) the concentration of substrate (glycogen), (2) the final product (glucose-6-phosphate) and by allosteric regulators that (3) positively interact (AMP) or (4) negatively interact (ATP). AMP and ATP are neither substrates nor end-products, they also do not serve as enzymatic cofactors and they therefore qualify as "allosteric" regulators (we return to this subject later on). AMP marks "starvation" as it is produced in a reaction that serves to maintain ATP levels ($2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$), whereas ATP marks plentitude. This way cells perfectly maintain their ATP levels around 5 mM.

However, in some situations, glucose-6-phosphate must be produced in excess, anticipating extreme muscular activity, for example, in the case of "fear, fight, or flight." Here is where adrenaline, known as the "stress hormone," comes into play. Adrenaline, through its signaling pathway, causes a covalent modification of glycogen phosphorylase, the addition of a phosphate at serine-14, which brings the enzyme in a highly active state (described as the "relaxed" state) and renders it less sensitive to glucose-6-phosphate or ATP (Figures 2-16–2-18). In short, adrenaline-mediated phosphorylation of serine-14 overrides the allosteric control that normally occurs through cellular metabolites.

The pathway that brings about this change has multiple steps. As we have already shown, it starts with receptor-mediated loading of the Gα-subunit with GTP, this leads to activation of adenylyl cyclase. The ensuing production of cAMP brings about a change in activity in yet another enzyme, protein kinase A. This occurs through binding of cAMP to an inhibitory subunit (RIα), a protein that normally blocks the catalytic site of the protein kinase. As a result of cAMP binding, the inhibitor changes conformation and detaches from the catalytic subunit. The liberated active protein kinase A transfers phosphates (a process referred to as phosphorylation) to a number of regulatory subunits of a big protein-complex named

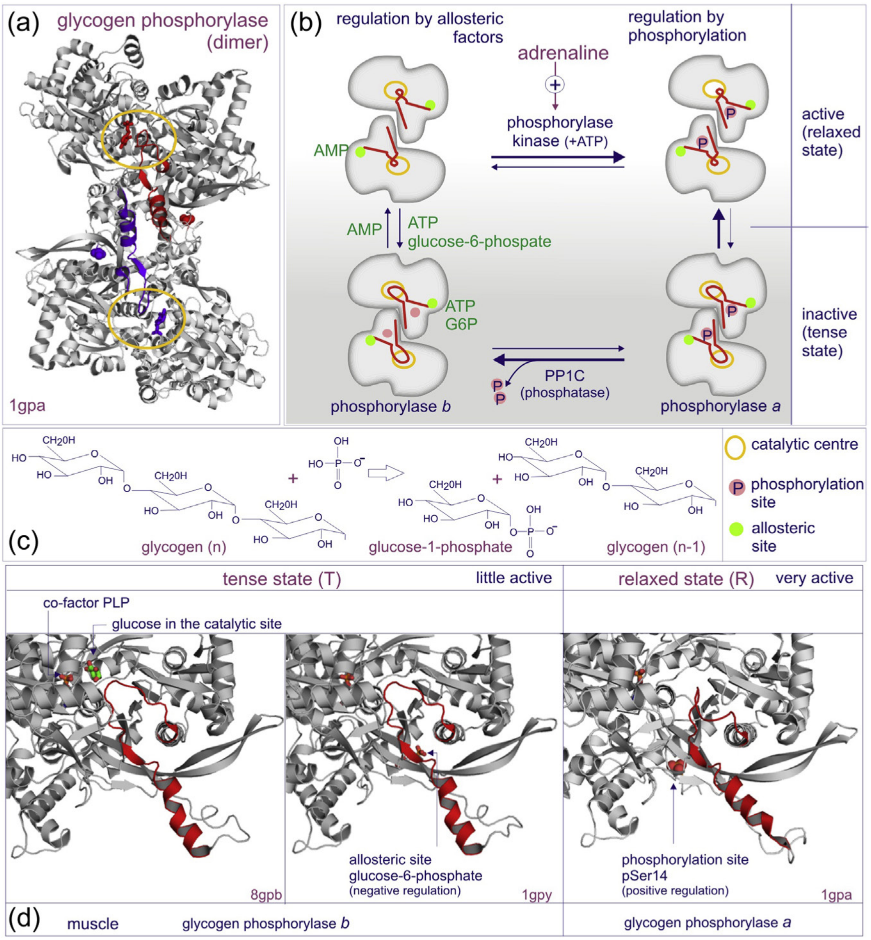


FIGURE 2-16 Regulation of glycogen phosphorylase by allosteric factors and phosphorylation. (a) Molecular structure of glycogen phosphorylase (forming a dimer) in which the position of the catalytic site is indicated by a yellow circle and the segment that undergoes conformational change is shown in red or blue. (b) Schematic representation of glycogen kinase and the effects of AMP and ATP (allosteric regulators), glucose-6-phosphate (G6P) and phosphorylation. Note that phosphorylation drives the kinase into the top right hand corner, a highly active "relaxed" state. (c) Detail of the phosphorylase reaction; at each step one glucose is removed from glycogen and one phosphate is added at position-C1. (d) Structural detail of glycogen phosphorylase highlighting the change in conformation of the amino acid loop that projects into the catalytic site.

phosphorylase kinase. By an as yet unclear mechanism this removes an inhibitory constraint from the catalytic subunit. The unique substrate of phosphorylase kinase is glycogen phosphorylase, which is phosphorylated on a single residue, serine-14 (Figure 2-17). Its phosphorylation particularly affects the positioning of a loop of nine amino acids situated in

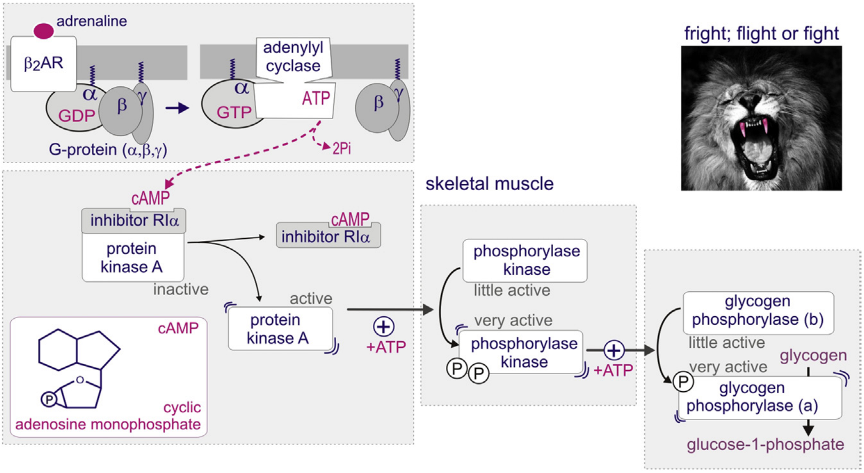


FIGURE 2-17 Schematic representation of the adrenaline to glycogen phosphorylase signaling pathway. The cascade starts with binding of adrenaline to its receptor, followed by loading of Gα (transducer) with GTP. This leads to activation of adenyl cyclase (effector) and subsequent formation of cAMP (second messenger). cAMP removes the regulatory subunit, allowing protein kinase A to express its activity. Phosphorylation of phosphorylase kinase ensues, causing the removal of an inhibitory constraint, and the cascade finally ends by phosphorylation of glycogen phosphorylase. The enzyme turns into a very active state and glycogen is degraded in numerous glucose-1-phosphates.

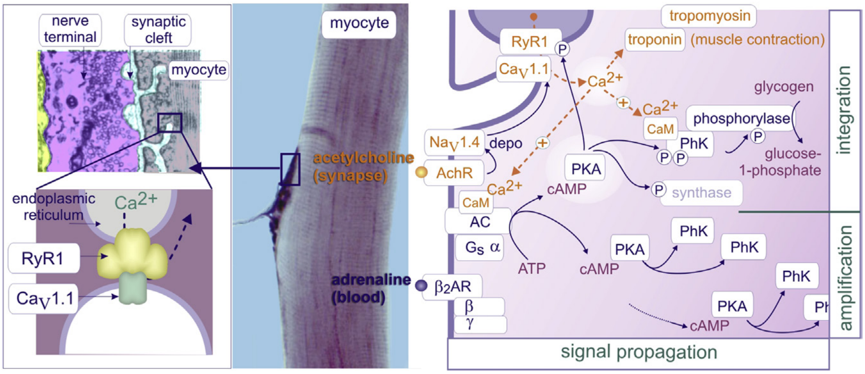


FIGURE 2-18 Pathway cross-talk between adrenaline and acetylcholine-mediated signaling events during muscle contraction. The second-messenger Ca^{2+} acts as an "interacting agent" as it stimulates both muscle contraction and elevates the activity of adenyl cyclase and phosphorylase kinase, both enzymes that operate in the β-adrenergic receptor pathway. In turn, PKA interferes with the cholinergic pathway by phosphorylating RyR1 which results in an enhanced flow of Ca^{2+} (enforcing muscle contraction). PKA also phosphorylates and inactivates glycogen synthase. PKA is said to constitute a signaling node. Signal amplification occurs through the catalytic action of adenylyl cyclase and PKA, the cyclase produces numerous second messengers and each protein kinase phosphorylates multiple substrates. In certain cases, kinase and substrate form a complex and they function in a 1:1 stoichiometry (case of PhK and glycogen phosphorylase). Abbreviations: PhK phosphorylase kinase; phosphorylase, glycogen phosphorylase; RyR1, ryanodine-sensitive IP_3 receptor; Ca_V and Na_V, voltage-dependent calcium and sodium channel, respectively; βAR, beta adrenergic receptor, AchR, nicotinic acetylcholine receptor, PKA, protein kinase A, Gs, stimulatory G-protein; CaM, calmodulin; cAMP, cyclic adenosine monophosphate.

the catalytic cleft. It changes from a "tense" to a "relaxed" state, thereby rendering the enzyme fully accessible to glycogen (and hence rendering the enzyme fully active) (molecular detail in figure 2-16).

Pathway cross-talk: nodes and interacting agents

Skeletal muscle contraction is controlled by motor neurons. They terminate on muscle fibers in an anatomical structure called the motor endplate. There they release acetylcholine that crosses the synaptic cleft and binds to its receptor, a Na^+/K^+ ion channel. The channel opens and in first instance Na^+ enters (driven by both a chemical and an electric gradient). This leads to a partial depolarization of the plasma membrane to which voltage-sensitive ion channels (NaV1.4) react by opening. A full depolarization ensues and this changes the conformation of two physically linked Ca^{2+} channels, $Ca_V1.1$ and RyR1, with a resulting flow of Ca^{2+} from the endoplasmic reticulum into the cytoplasm (Figure 2-18). The cytoplasmic Ca^{2+} binds to troponin, which is bound to tropomyosin, and this causes a displacement of the complex on the actin filament, leaving the way to myosin to interact with actin. The actin–myosin interaction sets off the ATP-hydrolysis cycle in the myosin head and thus initiates muscle contraction.

Intracellular free Ca^{2+} not only binds troponin (calmodulin-like protein involved in muscle contraction) but also binds calmodulin (CaM) associated with adenylyl cyclase and with phosphorylase kinase (constituting the delta-subunit of the phosphorylase complex). Binding of Ca^{2+}/calmodulin augments their enzymatic activity even further and thus enforces the adrenaline pathway. The two pathways, adrenaline and acetylcholine mediated, are said to act in synergy and Ca^{2+} acts as an "interacting agent" (Figure 2-18).

Cross talk can also occur when a protein kinase interacts with more than one substrate thereby branching out into other terrains. Within the context of muscle contraction, PKA phosphorylates and inhibits glycogen synthase, an enzyme which is activated by insulin in the process of glucose removal from the blood. By inhibiting glycogen synthase PKA prevents that glycogenolysis and glycogen synthesis operate simultaneously (which makes no sense). PKA has also been shown to phosphorylate the above-mentioned RyR1 receptor on ser-2844 (mouse) and this enhances the Ca^{2+} flow and as a consequence the contractile force of the muscle (a phenomenon known as inotropy) (Andersson et al., 2012). In this particular setting PKA can be considered as a signaling node, it relays signals to different effectors. The current thinking is that all pathways will have interactions with other pathways; cross-talk through signaling nodes and agents is the rule rather than the exception. It should be noted that not all branches are necessarily essential for the outcome of the response and "indiscriminate chatter" between kinases and phosphatases may occur (Levy et al., 2009; Breitkreutz et al., 2010) in a way similar as that observed for transcription factor–DNA-binding networks (Euskirchen and Snyder, 2004).

FIGURE 2-19 Krebs (left) and Fischer in their former lab space, the morning of the Nobel Prize announcement (University of Washington, Seattle, 1992). With respect to the cheerfulness, Eddy Fischer wrote us "The picture was taken the day the prize was announced at the time Bill Catterall was showing the lab in which Ed and I, working side-by-side, had started our studies on phosphorylase. I noticed that they had replaced the large yellow ceramic sink that existed, by a stainless-steel one. When Bill said they had thrown away the old one, I told him he should have broken it up in pieces and sold the pieces, just like people had sold pieces of the Berlin wall."

A "Nobel" pathway

The above-described events have given rise to no less than 10 Nobel prizes. Awarded in 1947 to Carl Cori and Gerty Cori-Radnitz (for their discovery of the mechanism of glycogen breakdown), in 1970 to Luis Leloir (for his discovery of sugar nucleotides and their role in the biosynthesis of carbohydrates), in 1971 to Earl Sutherland (for his discovery of the second-messenger role of cAMP), in 1992 to Edwin Krebs and Edmond Fischer (for their discovery of phosphorylation as a means to modify protein activity) (Figure 2-19), in 1994 to Alfred Gilman and Martin Rodbell (for their discovery of GTP-binding proteins as transducers in signaling), and in 2012 to Robert Lefkowitz and Brian Kobilka (for their studies on G-protein-coupled receptors). Read more about their work at www.nobelprize.org. Moreover, not only Nobel but noble too, because Philip Cohen received a knighthood in 1998 for his work on protein phosphorylation, which in the early days focused on the composition of phosphorylase kinase and the role phosphatases (PPP1CA) and glycogen synthase kinase (GSK3) in the regulation of glycogen metabolism (Cohen, 2009). Louise Johnson received a knighthood in 2000 for her work on structure function analysis, in which the elucidation of molecular changes in glycogen phosphorylase (Sprang et al., 1988) and mechanisms of kinase activation played an important part.

Web resources

For more information about the structure of glycogen phosphorylase we refer to “Protein of the Month” (RCSB Protein Data Bank): http://www.rcsb.org/pdb/101/motm.do?momID=24.

WIRED ALLOSTERY AND THOUGHTFUL DECISIONS

The above-described pathways show a multitude of mechanisms that lead to a cascade of shape changes, in other words, a cascade of “allostery.” Among these are: (1) ligands binding to receptors, (2) an engaged-receptor interacting with a G-protein, (3) an exchange of GDP for GTP, (4) membrane depolarization affecting voltage-sensitive ion channels, (5) second messengers binding to regulatory subunits (cAMP binds RI, Ca^{2+} binds troponin and calmodulin), and (6), last but not least, protein-kinases phosphorylating proteins. In our example, the cascade terminates with the activation of glycogen phosphorylase and the contraction of muscle. The cascades constitute “wires” that connect receptors with effectors (those that effectuate the cellular response). In the next section, we return to the numerous mechanisms that cells employ to bring about shape changes or changes in subcellular localization of components of signaling cascades.

The questions arise: why can there not be a direct connection between the receptor and the ultimate effector enzyme? That would save a lot of memory in learning signaling events. And why would pathways want to crossover as it only confuses matters further? The answer is that pathways have not been designed; they came about, either in a simple or seemingly complicated manner, and proved to be sufficiently robust to stand the test of time. There are however strong arguments in favor of signaling cascades and these are: signal propagation deep into the cell, signal amplification, and, most importantly, enhanced signal integration (Figure 2-18). Signal propagation into the cell is essential as not all cellular events take place at the plasma membrane. Many signaling events will attain the nucleus and this requires a translocation; the movement of proteins from one cellular compartment to another. The aspect of amplification is easy to grasp, one adenyl cyclase produces numerous copies of cAMP; these stimulate numerous copies of protein kinase A, each of which can in turn phosphorylate numerous copies of phosphorylase kinase. It must be noted however that not all cascades allow for stepwise amplification because in certain cases the components are confined to a “cassette” at 1:1 stoichiometry (the protein kinase has access to only one substrate). Enhanced signal integration is the consequence of a multitude of opportunities to bring about shape changes. If there was a direct connection between the receptor and glycogen phosphorylase, Ca^{2+} could not have taken part in the regulation of its activity. Of course one could correct this shortcoming by inventing a calmodulin-binding site on glycogen phosphorylase, but this has not happened naturally. Moreover, if pathway design would follow the logic of short connections, in order to allow for the numerous allosteric agents to have their say, it would probably bring about a list of near impossible constraints on the composition of proteins. Take the example of glycogen phosphorylase: it has to be sensitive to both intracellular (AMP, ATP, glucose-6-phosphate) and extracellular (anticipating extreme labor) conditions, it has to be located at the membrane in order to receive extracellular signals (acetylcholine- and adrenaline receptors) but, at the same time, also being able to reach glycogen storage-granules to bring about glycogenolysis. As a last argument to explain complexity, signaling pathways employ the same core components for a multitude of events; cells follow a "modular approach" to pathway construction. Among other advantages, a modular approach reduces the number of different proteins required to perform different functions (Gerhart and Kirschner, 2007).

Integration of multiple signaling pathways, comprising those that reflect extracellular as well as intracellular conditions, constitutes the "thoughtfulness" of cellular communication. Multiple interests are balanced at multiple sites in the pathways and (in analogy with neuronal decision taking as we understand it today) this opens the way to "thoughtful" decisions (Breitkreutz et al., 2010), (Grand, 2003). Enhanced networking of proteins is a key characteristic of increasingly complex organisms (return to Figure 1-7 in Chapter 1) (Anantharaman et al., 2007).

ALLOSTERY

The term allostery just means "other space" or "other shape." The word allosteric was coined to qualify the mechanism of feedback inhibition exerted on bacterial regulatory enzymes by their regulatory ligands (Changeux, 1961; Monod et al., 1963). In contrast to the classical mechanism of inhibition by mutual exclusion due to steric hindrance (for instance through an antagonist), the allosteric mechanism for feedback inhibition takes place between nonoverlapping, stereochemically distinct, sites for substrates, and regulatory ligands. Such interactions mediated by discrete reversible alterations of the molecular structure of the protein (or change in the relative position of subdomains) were named allosteric interactions. The concept of allosteric regulation became the subject of numerous studies after publication of an article, in 1963, by Monod, Changeux, and Jacob, entitled "allosteric proteins and cellular control systems" (Monod et al., 1963). The original model relies on two main principles: the occurrence of a symmetrical oligomeric

ALLOSTERY *(cont'd)*

structure, which is indeed the case for most signal-transducing proteins, and a preexisting conformational equilibrium, differentially shifted and stabilized by allosteric factors. Allostery occurs probably nearly everywhere: receptors, protein kinases, metabolic enzymes, cytoskeletal components, transcription factors, and why not at the level of nucleotides (they too fold into complex tertiary structures). Today, allosteric regulation is no longer restricted to the action of small metabolites, nor to symmetrical oligomeric structures, as in 2006 the term was also employed to explain the activation of the protein kinase domain of the EGF receptor, which occurs through the formation of an asymmetric dimer in which one kinase modifies the shape of the other (much like cyclins activating the cyclin-dependent kinases) (Zhang et al., 2006). We consider covalent modifications, for instance phosphorylation, as yet another way of bringing about allostery. More recently, a whole new field of allosteric regulation was made apparent with the finding that intrinsically disordered proteins can bind to folded protein and create novel binding sites that, in turn, cause the assembly of unique protein complexes that influence cell functioning. Examples of intrinsically disordered cellular proteins that can interact with a large range of partners are p53 and BRCA1 (Uversky et al., 2008). The intrinsically disordered E1A protein of adenovirus E1A causes genetic reprogramming by binding to different cellular partners (Ferreon et al., 2013).

For further reading about a more purist approach to allostery we refer to: Changeux, J-P., 2012. Allostery and the Monod–Wyman–Changeux Model after 50 years. Annu. Rev. Biophys. 41, 103–133.

POSTTRANSLATIONAL MODIFICATIONS INVOLVED IN SIGNALING EVENTS

Nucleotide exchange and phosphorylation

As already mentioned, two mechanisms bring about switches in signaling events: a change in activity and a change in subcellular localization. Both mechanisms lead to changes in the "wiring" of proteins. The two may be interdependent; translocation may alter conformation of a regulatory subunit, and hence activate an enzyme, or bring an enzyme to its substrate. Within the context of receptor-mediated signaling discussed above, the switches are caused by an exchange of nucleotides, by the intracellular release or production of second messengers such as Ca^{2+} and cAMP, and

TABLE 2-3 Covalent modifications involved in changing protein activity or subcellular localization

Modification	Source	Example
Phosphorylation	ATP	Glycogen phosphorylase (activation)
Acetylation	Acetyl CoA	Histone (reduction in DNA and histone binding)
Methylation	S-Adenosylmethionine	FOXO1 (inhibition of phosphorylation by PKB)
Myristoylation	Myristoyl CoA	C-abl (inhibition of kinase activity)
ADP ribosylation	NAD	RNA polymerase (transcription)
Farnesylation	Farnesyl pyro-Pi	Ras (membrane localization)
Gamma-carboxylation	$HCO_3{}^-$	Thrombin (clotting)
Sulfation	Phosphoadenosine or phosphosulfate	Fibrinogen (clotting)
Ubiquitinylation	Ubiquitin	Traf6 (docking site for signaling complex)
Polyglutamylation	Glutamate	Tubulin (severing of microtubules)
N-acetylglucosamination	UDP-GlcNAc	Nuclear pore protein p62 (RNA transport)
Nitration of tyrosine	Peroxynitrite ($NO + H_2Oh_2$)	EGF receptor (dimerization)
Oxidation of cystein thiolate	H_2O_2	PTP1B tyrosine phosphatase (inactivation)

by the covalent attachment of a phosphate (posttranslational modification of protein). The list of possible covalent posttranslational modifications is long and growing (see Table 2-3) (Yang, 2005; Hunter, 2007). Not all have necessarily come to light in signaling events and they are not necessarily related to receptor action (not yet). Below we highlight three examples: the role of ubiquitinylation in the formation of the TNFα-receptor signaling complex, the role of myristoylation in the regulation of the tyrosine kinase (Abl), and we show that multiple modifications occur on Histones. Further down we will elaborate extensively on nucleotide exchange and protein phosphorylation, two modifications intimately related to signal transduction.

Ubiquitinylation

Tissue necrosis factor-α (RNFα) is a key mediator of inflammation because it leads to enhanced expression of adhesion molecules on endothelial cells and thus to the recruitment of immune cells into the tissues (both cells that belong to the innate and the adaptive immune system). In this context its principal receptor is TNFR1. Both ligand and receptor are expressed as trimers. When TNFα binds TNFR1, a protein complex attaches to the cytoplasmic domains. These may all be considered to act as adaptor proteins as they do not manifest enzymatic activity. Their role is to recruit yet another protein complex that has the capacity to transfer a chain of polypeptides, called ubiquitins, onto a lysine residue on RIP1 (Figure 2-20). The RIP1-bound chain of ubiquitins has an unusual linkage, involving lysine-63 (K63) instead of lysine-48 (K48). This is an important

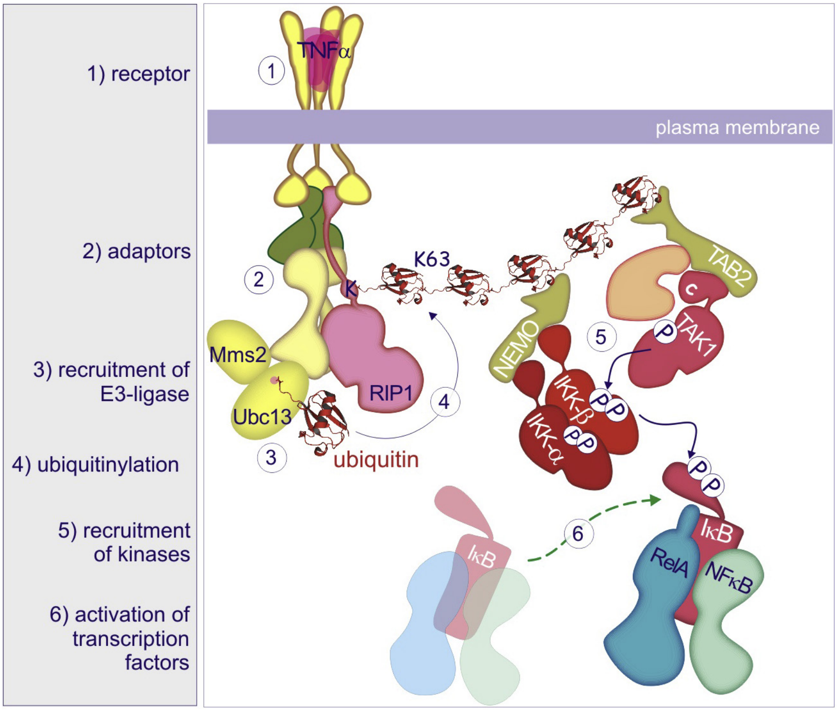


FIGURE 2-20 Ubiquitinylation as a means to recruit signaling complexes. Receptor trimerization leads to the recruitment of adaptors, which in turn recruit components of the ubiquitinylation machinery. As a consequence RIP1 gets poly-ubiquitinylated on lysine in a K63-fashion. The ubiquitin chain serves to bind new adaptors NEMO and TAB2, which carry with them a set of protein kinases. A cascade of "activating" phosphorylations follows that ultimately lead to activation of the transcription factors RelA and NFκB.

distinction because normally, ubiquitinylation serves to mark proteins for destruction but the K63-linkage is not recognized by the proteasome (protease complex). Instead, the "K63-chain" acts as a docking site for adaptors (NEMO and TAB2) which are associated with protein kinases. The ubiquitins have the role of activating the protein kinase IKK-β, by bringing it in contact with two other protein kinases namely TAK1 and IKK-α. The activated IKK-β finally brings the signal into the cell by liberating the transcription factors NFκB and RelA from their shared inhibitor. The liberated transcription factors now translocate to the nucleus there to alter gene transcription (enhanced expression of adhesion molecules but also chemokines and enzymes involved in the production of prostaglandins).

Lipidation

Cytosolic proteins may become intrinsic membrane proteins through the covalent addition of fatty acids and/or isoprenoids (collectively named lipid tails). For instance, the heterotrimeric G-protein complex is attached to the membrane this way: two subunits are connected with palmitate (also referred to as a palmitoyl group). In certain cases, however, the lipid tail can be buried in a hydrophobic pocket within the protein or within another protein. This not only affects protein localization, being membrane associated or not, but may also affect protein structure and activity. In the case of the protein recoverin (RCVRN), present in photoreceptors of the retina, the second-messenger Ca^{2+} dictates both its subcellular localization and its activity. In the absence of Ca^{2+}, the myristoyl group is concealed in a pocket within the protein. In the presence of Ca^{2+}, the myristoyl is ejected from its pocket and the protein associates with the membrane. The empty pocket now interacts with and inactivates rhodopsin kinase (GRK1), which is also a membrane-attached protein (through a farnesyl group). This change in subcellular localization occurs at low light levels (Zozulya & Stryer, 1992; Ames et al., 2012) and it prevents deactivation of the GTP-binding proteins by phosphorylation of the receptor. When light levels increase, intracellular Ca^{2+} levels rise and recoverin returns to its "inactive" state, detached from rhodopsin kinase, which now phosphorylates and inactivates the photoreceptor (rhodopsin) so that the system rapidly resets (in order to be reactivated by the next wave of photons) (Figure 2-21(a)). Another example is the tyrosine protein kinase C-Abl. It has a myristate tail, but the protein kinase is not normally membrane bound. The myristate serves to control its activity. C-Abl is a tyrosine protein kinase, it transfers phosphate on tyrosine residues. It occurs both in the cytoplasm and in the nucleus. In its inactive state, the myristate is concealed inside the protein kinase and in doing so it pulls two regulatory domains (SH2 and SH3) tightly against the catalytic domain leading to a distortion that prevents activity (a state referred to as "latched conformation"). Ejection of the myristate, which

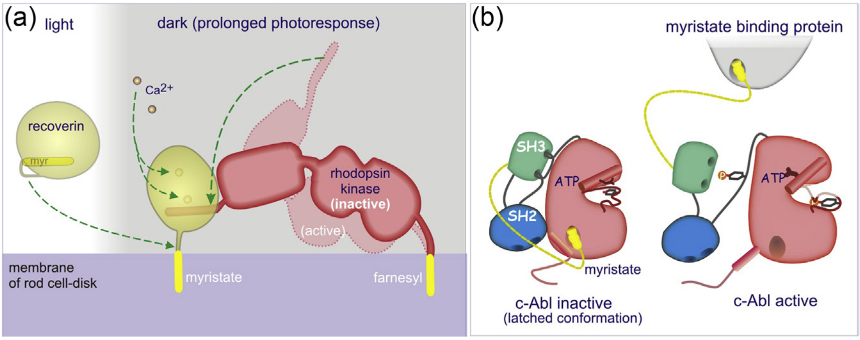

(a)
light
dark (prolonged photoresponse)
Ca^{2+}
recoverin
myr
rhodopsin kinase (inactive)
(active)
membrane of rod cell-disk
myristate
farnesyl
(b)
myristate binding protein
SH3
SH2
ATP
myristate
c-Abl inactive (latched conformation)
P
ATP
c-Abl active

FIGURE 2-21 The role of myristoylation in control of subcellular localization and/or activity. (a) Recoverin, a protein expressed in rod cells of the eye, carries a myristate chain (fatty acid) which switches between a protein- and membrane-bound state. When intracellular Ca^{2+} is low in the rod cell, which occurs in the presence of light, the myristate resides inside the protein. Increased levels of Ca^{2+}, which occurs in the dark, chases the myristate out of its pocket and it now buries itself in the membrane of the photoreceptor disk. The empty pocket is next occupied by a C-terminal tail of rhodopsin kinase and this leads to kinase inactivation. (b) The tyrosine protein kinase c-Abl holds itself in an inactive state through a latched conformation in which myristate inserts into a pocket within the protein kinase (on the left). The presence of a myristate-binding protein allows the myristate to switch host and this relieves the latch. The kinase will need to undergo two other modifications in order to achieve full activity.

occurs when the myristate is concealed by another myristate-binding protein, releases the inhibitory constraint and allows further processing that turns the protein kinase to a fully active state (Figure 2-21(b)).

Acetylation, crotonylation, and methylation

DNA is highly compacted and organized into chromatin by both histone and nonhistone proteins (for instance the high mobility group proteins (HMGs)). The basic unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped 1.6 times around an octamer of core histone proteins (H2A, H2B, H3, and H4). These four canonical histone-proteins are composed of a structured central (globular) domain that is in close contact with the DNA and a less well-structured amino-terminal tail domain. Histones undergo abundant posttranslational modifications in both their globular and tail domains (see Figure 2-22). Several histone modifications have been identified, including: acetylation, crotonylation, methylation, phosphorylation, and ubiquitylation. The combination of such histone modifications (the "histone code") is indicative of whether a gene is transcriptionally active or inactive (Yun et al., 2011; Goll and Bestor, 2002; Lee and Workman, 2007; Grant, 2001). Collectively, histone modifications make an important contribution to what we call epigenetic regulation of the genome. Histone modification can be passed to daughter cells as epigenetic marks.

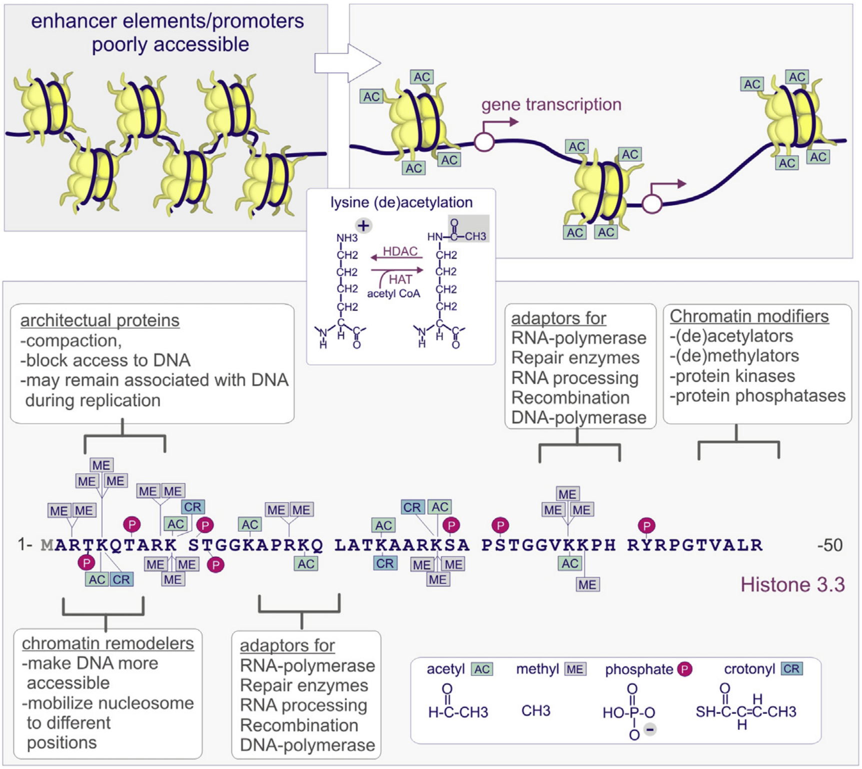


FIGURE 2-22 Acetylation, methylation, crotonylation, and phosphorylation constitute the "histone code" for recruiting enzymes to the DNA.

All histones may be acetylated on specific lysine residues in their amino-terminal domain (examples in Figure 2-22). An important consequence of acetylation is the removal of the positive charge of lysine that leads to a reduced electrostatic contact with the DNA and with neighboring nucleosomes. This in turn gives rise to a more flexible chromatin where the DNA is more accessible and thus amenable to transcription. In a cellular context, the level of acetylation specifies geographically euchromatin (richly acetylated) and heterochromatin (poorly acetylated). Acetylation also acts as a docking site for proteins (see below for methylation). Methylation may occur on lysine or arginine. These methylated residues act by creating docking platforms for adaptors that, in turn, recruit enzyme complexes to the DNA. Depending on the methylation pattern these include: transcription factors, double-strand repair machinery, chromosomal recombination enzymes, RNA splicing regulators, or the DNA-replication machinery (Yun et al., 2011). Crotonylation occurs on lysine residues and it marks active promoters and confers resistance to transcriptional repressors. It is also associated with postmeiotically activated genes on autosomes (Tan et al., 2011).

Finally, histones are also subject to phosphorylation and this may serve to orchestrate subsequent acetylations, methylations, or crotonylations. Phosphorylation of histone, in addition to phosphorylation of transcription factors that also play a role in recruiting acetylation/deacetylation enzymes to the DNA, may constitute an important connection between epigenetic events and the cell internal and external environments (Liokatis et al., 2012; Rossetto et al., 2012; Bode and Dong, 2005; Edmunds and Mahadevan, 2004; Mahadevan et al., 1991).

EPIGENETICS

The above-mentioned histone modifications can be passed to daughter cells. This may, for instance, serve to preserve lineage specificity within stem cell populations. The modifications of histones and DNA (methylation of cytosine) are called "epigenetic" as they affect gene transcription and thus phenotype without affecting gene sequences. Conrad H. Waddington coined the term 'epigenetic landscape' in 1942, in order to explain how environmental signals (coming from neighboring cells) could interfere with development. This process involves turning-on as well as turning-off genes that are involved in cell fate determination. His theories have brought new impetuous to developmental research in the post-Spemann period. Hans Spemann, who received a Nobel Prize in 1935, had postulated the presence of organizing centers in the developing animal but without giving many clues as how they could determine cell fate (Waddington, 1942). In the words of Waddington: "One might say that the set of organizers and organizing relations to which a certain piece of tissue will be subject during development make up its 'epigenetic constitution' or 'epigenotype'; then the appearance of a particular organ is the product of the genotype and the epigenotype, reacting with the external environment." (for more information: http://www.ehudlamm.com/epigenotype.pdf).

Web resources

- "dbPTM," an informative resource for protein posttranslational modification (National Chiao Tung University)http://dbptm.mbc.nctu.edu.tw/index.php.
- "PhosphoSitePlus," a protein modification resource (Cell Signaling Technology) www.phosphosite.org.
- "uniprotKB," Universal Protein Resource (EMBL-EBI, PIR, SIB) http://www.uniprot.org/.

FOCUS ON NUCLEOTIDE EXCHANGE

G-proteins are transducers

G-proteins, or GTP-binding proteins or GTPases, function downstream of the majority of receptors but we must make a distinction between two different types; the monomeric and heterotrimeric G-proteins. Monomeric G-proteins were discovered as single small proteins, comprising roughly 180–300 amino acids, hence commonly referred to as small GTPases. The heterotrimeric G-proteins were discovered as complexes of three subunits, α, β, and γ, of which only the α-subunits (Gα) bind GTP (the β and γ do not classify as members of the G-protein family). From an evolutionary perspective, heterotrimeric G-proteins have evolved from monomeric ones. The term GTPase indicates that G-proteins have the capacity to hydrolyze GTP into GDP and Pi. However, the intrinsic GTPase activity of monomeric G-proteins is too little to speak of, and necessarily they require assistance, whereas heterotrimeric G-proteins can hydrolyze GTP without help. Whereas monomeric G-proteins cover nearly all functions of the cell (ranging from nuclear transport, protein synthesis, vesicle formation, to signaling from growth factor receptors) and are controlled by a large variety of mechanisms, the heterotrimeric G-proteins (Gα) are (nearly) all directly controlled by seven-transmembrane-spanning receptors (G-protein-coupled receptors of GPCRs) and function almost exclusively in cell membrane-operated signaling mechanisms (receptor type-2 in Figure 2-13).

G-proteins share a remarkable set of highly conserved amino acids, the G-boxes, which are involved in nucleotide binding and interaction with effectors (switch regions). Five such conserved sequences have been identified, named G1-5, and in Figure 2-23 we provide three examples of G-boxes of three rather distinct G-proteins: HRas (monomeric), Gαi (heterotrimeric), and prokaryotic EF-Tu (translation elongation factor from *Thermus aquaticus*, a G-protein that binds the amino–acyl-binding region of tRNA) (Bourne et al., 1990; Colicelli, 2004).

The G-proteins involved in receptor-mediated signaling events all belong to the RAS superfamily (Tables 2-4), which comprises 170 member separated over 5 (sub)families, namely: Arf, Gα, Rab, Ras, and Rho (ranked in alphabetical order). Gα makes an exception in this list in that it has a large insert, the alpha-helical domain (AH) (Figure 2-23). However, structural investigation reveals that this domain does not really alter the basic structure of the nucleotide-binding segment. As shown in Figure 2-24, Gα-subunits possess in fact two independent domains, the Ras-domain (sometimes referred to as the GαRas) and the alpha-helical domain (Dohlman and Jones, 2012). The alpha-helical domain covers the nucleotide-binding site on the GαRas domain and, as we will see later, must be displaced to allow GDP dissociation. Not surprisingly this

box	residues	sequence	function
G1	HRas (10-17) Gαi (40-47) EF-Tu (19-26)	GaggvGKS GagesGKS GhvdhGKT	P-loop or Walker A motif, purine nucleotide binding
G2	HRas (32-36) Gαi (178-182) EF-Tu (60-64)	ypdTi rvkTt rgiTi	Mg^{2+} coordination, γ-phosphate, in effector loop (switch 1)
G3	HRas (57-60) Gαi (200-2003) EF-Tu (82-86)	DtaG DvgG DcpG	Mg^{2+} coordination, glycine binds to γ-phosphate of GTP, in effector loop (switch 2)
G4	HRas (116-119) Gαi (269-272) EF-Tu (137-140)	NKcD NKkD NKvD	confers specificity for guanine over adenosine
G5	HRas (144-146) Gαi (324-326) EF-Tu (174-176)	tsA tcA gsA	buttresses purine nucleotide binding (G1)

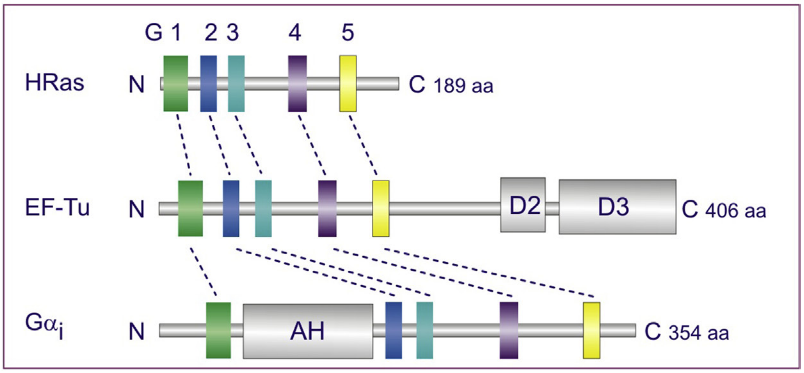


FIGURE 2-23 Conserved sequences involved in GTP-binding, the so-called G-boxes, in G-proteins. AH, alpha-helical domain, D2 and D3, EF-TU domain-2 and -3.

domain contributes to nucleotide binding and assists in hydrolysis of GTP (Markby et al., 1993).

Other types of G-proteins exist, roughly 50 proteins, and these carry recognizable G1, G3, and G4 boxes but are generally larger proteins due to the presence of additional functional domains. Examples are the dynamins (intracellular vesicle fission), the initiation and elongation factors (protein synthesis), the signal-recognition particle receptor (routing of ribosome to the rough ER), the septins (cytokinesis), and, very distantly related, the β-subunit of tubulin (microtubules).

Many G-proteins of the RAS superfamily are subject to lipid modifications which promote association with cellular membranes (or with proteins of the GDI family). Attachment of farnesyl or geranylgeranyl

TABLE 2-4 Members of the RAS superfamily of G-proteins, their GEFs and GAPs, lipid modifications, and broad functions (Offermanns, 2003; Cherfils and Zeghouf, 2013; Siderovski and Willard, 2005)

Gene family	Some members	Covalent modification	GEFs/GAPs	Broad functions (among others)
Arf/Sara (30 members)	ARF1 ARL6 SARA1A (Sar1)	Myristate or palmitate (fatty acids) (no known GDI)	• ArfGEF with Sec7 domain (16 members) • Sec23/Sec13 specific for Sara1 • ArfGAP with ArfGAP domain (31 members) • RP2 (Arl3GAP) regulators of ARL remain elusive	Major regulators of almost every aspect of membrane traffic • Recruitment of vesicle coat proteins • Sorting cargo for primary lysosome in *trans*-Golgi • Vesicle budding in ER ("exit-sites") and in Golgi • Microtubule-mediated vesicle transport
Gα (16 members)	GNAS (GαS) GNAT (Gαt1) GNAL (Gαolf)	Myristate or palmitate	• GPCR receptors (800 members) • AGS1 (1) • Ric-8 (1) • RGS proteins (25 members) • Effectors proteins: PLCβ, AC, cGMP-PDE, RhoGEF	Signaling downstream of GPCR receptors
Rab/Ran (71 members)	RAB5A RAN	Farnesyl or geranylgeranyl (isoprenoids) RabGDI	• RabGEF with Vsp domain (10 members) • RabGEF with DENN domain (18 members) • Component of TRAPP complex (Rab-specific) • SERGEF (Ran-specific, with RCC1 repeats) • RabGAP with TBC domain (40 + members) • Rab3GAP • RanGAP	RAB • Recruitment of vesicle coat proteins • Fusion of endocytotic vesicles into endosome • Receptor uptake (EGFR) RAN • Nuclear transport

Ras (35 members)	KRAS RALA REM RAP1A RHEB	Farnesyl or geranylgeranyl (isoprenoids), for some, myristate palmitate	• RasGEF with Cdc25-homology domain (27 members) • EPAC, rapGEF sensitive to cAMP • RasGAP active on all (15 members) • RapGAP active on Rap/Ral (11members)	• Growth factor signaling • Cell motility • Cell attachment • Protein synthesis (RHEB)
Rho (22 members)	CDC42 RAC1 RHOA	Farnesyl or geranylgeranyl, for some, myristate palmitate 3 RhoGDIs	• RhoGEF with Dbl-homology domain (70 + members) • RhoGEF with dock-homology region (11 members) • GAP with unique RhoGAP domain (70+ members)	• Organization of the actin cytoskeleton • Filopodia • Lamellipodia • Stress fibers • Podosomes/invadopodes

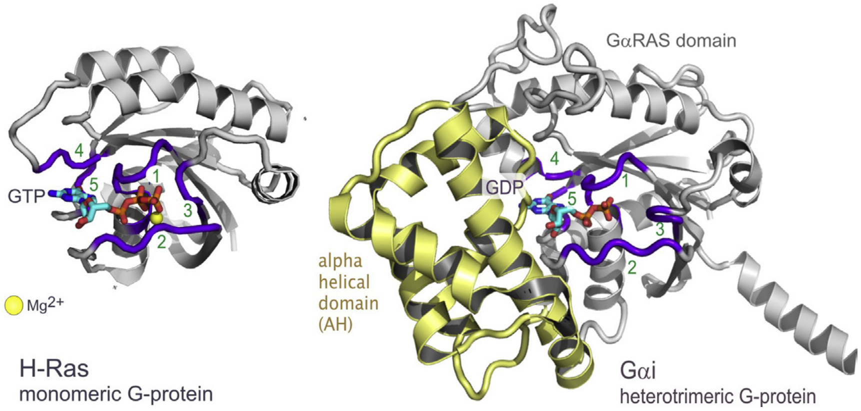


FIGURE 2-24 Topography of highly conserved amino acids (G-boxes 1, 2, 3, 4, and 5) in G-proteins. Left, example of the H-Ras and right, the Gα-subunit of the heterotrimeric GαI (see Figure 2-24 for corresponding amino acid sequences). pdb:1lf0, 1gg2).

(isoprenoids) to the C-terminal cysteine is observed for Rab, Ras, and Rho family members. For certain Ras and Rho members a palmitate (fatty acid) is covalently linked to the cysteine. Members of the Arf and Gα families are modified by attachment of myristate and/or palmitate (both fatty acids) on their N-terminal glycine (Table 2-4).

ATP IS NOT QUITE WHAT IT SEEMS

The role of GTP in signal transduction became apparent in the years following the discovery of cAMP (Rall et al., 1957) when scientists studied the connection between hormones and activation of adenylyl cyclase. All that was required (beyond a great deal of skill and dedication) to detect cAMP in cell membrane preparations was an activating hormone and ATP (as its Mg^{2+} salt). The membranes, containing an appropriate receptor and the catalytic unit, did the rest. At least that was the general idea. Around 1970, when the production of ATP became more and more industrial, the outcome of the experiments became erratic, occasionally registering zero activity in the cyclase reaction. A similar impasse had been encountered previously in the investigation of fatty-acid biosynthesis; an essential impurity was lost as the chemical purity of the ATP was improved. In the case of fatty-acid synthesis this occurred to be CTP and with respect to activating adenylyl cyclase it proved to be GTP. Only later did it appear that this GTP "cofactor" acted through binding to the "transducer," the GTP-binding protein that connects receptor and effector (Rodbell et al., 1971; Gilman, 1987).

The GTPase cycle

G-proteins typically exist in two states, an active GTP-bound state and an inactive GDP-bound state. Like for receptors, the active state is defined as the state in which the protein interacts with downstream effectors. The cycle of events common to all G-proteins starts and ends with GDP situated in the guanine nucleotide-binding pocket. Upon interaction with a guanine exchange factor (GEF), which in the case of heterotrimeric G-proteins is an occupied 7TM-receptor (Oldham and Hamm, 2008), the nucleotide-binding pocket is opened and the binding of GDP is weakened. As a consequence GDP dissociates. It is naturally replaced by GTP because this is present at a ~10-fold higher concentrations in the cell and binds with higher affinity to the nucleotide pocket (because of an additional phosphate). As a consequence, two or three switch regions, for mono- and heterotrimeric G-proteins respectively, change shape and the contact with the guanine nucleotide exchange factor is weakened. The monomeric G-proteins directly seek contact with effectors whereas the Gα-subunit first dissociates from the heterotrimeric complex (case for $G\alpha_s$) (Janetopoulos et al., 2001) or, alternatively, remains attached but reorients its position relative to Gβγ (case of $G\alpha_i$) (Bünemann et al., 2003), and then seeks other partners. The βγ-subunits also seek other partners (independently or associated with Gα). In conclusion, the shift from a GDP- to a GTP-bound state brings about a change in configuration which, as a consequence, alters the connectivity of the G-proteins (and of their βγ-partners). After some time, in the order of milliseconds to tens of seconds, the GTP is hydrolyzed upon interaction with a GTPase-activating protein (GAP) and the G-protein returns to a GDP-bound, inactive, state. Certain G-proteins (members of Rab and Rho families that carry a farnesyl or geranylgeranyl group in their C-terminus) then bind to an inhibitory protein, guanine dissociation inhibitor (GDI), whereas heterotrimeric Gα-proteins re-associate with Gβγ to reconstitute trimeric complexes. In the case of Gαi, it can also bind to AGS1 proteins (proteins that function as a GDI). Association with GDI lengthens the GDP-bound inactive state as it prevents interaction with GEFs (Cherfils and Zeghouf, 2013; Siderovski and Willard, 2005) (Figure 2-25).

With respect to the monomeric G-proteins, the majority of GAPs act by inserting a positively charged arginine-finger into the nucleotide pocket, near the 3rd (γ) phosphatase of GTP (Oldham and Hamm, 2008). This neutralizes the negative charges of the γ-phosphate and allows a transfer of phosphate to water (see textbox "nucleophilic substitutions to phosphorus", page 123). The α-subunits of heterotrimeric G-proteins (Gα) already possess this arginine, and indeed they spontaneously hydrolyze GTP much faster as the monomeric ones. However, GTPase activity is still controlled by GAPs and they function by correctly positioning the water molecule so that the phosphate can be transferred (Tesmer et al., 1997).

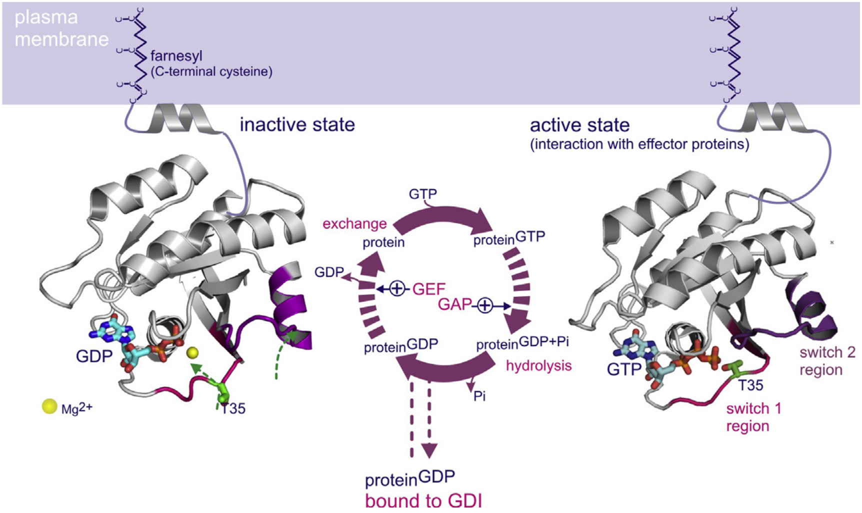


FIGURE 2-25 The GTPase cycle starts with the G-protein in its GDP-bound state. Under the influence of a guanine exchange factor (GEF) the GDP is exchanged for GTP and this brings about conformational changes in the so-called switch regions (compare the left with the right image of H-Ras, the shift of threonine-35 in the switch-1 region is highlighted). The G-protein is said to be in its active state, ready to interact with effectors. After some time, in the order of milliseconds to tens of seconds, a GTPase-activating protein catalyzes the hydrolysis of GTP into GDP and Pi and the G-protein returns to its GDP-bound inactive state. The H-Ras protein is attached to the membrane with a farnesyl chain linked to the C-terminal cysteine (S-farnesyl cysteine).Pdb: 1 lf5, 1lf0.

With respect to the GTPase cycle for heterotrimeric G-proteins, this is best described as the timed light-switch in communal staircases of apartment blocks, the kind which, when pressed (GDP/GTP exchange), switches the light for just the time it takes to get to the next landing (when GTP hydrolysis occurs). In electronic parlance this would be called a *monostable switch.* The additional helix-domain insert (in between the G1 and G2 box) of the Gα family lowers the intrinsic exchange activity but elevates the intrinsic GTPase activity (Markby et al., 1993). As already mentioned, monomeric G-proteins have both very low intrinsic exchange and hydrolysis activity. Without GEFs and GAPs it is estimated that for N-Ras, both the exchange of nucleotide and subsequent hydrolysis of GTP would only occur once per hour (Neal et al., 1988). Indeed, mutations in Ras that hinder access to the arginine finger of Ras-GAP (mutations G12V and G13D) render these G-protein constitutively active, resulting in a lack of control of cell differentiation and proliferation (Pylayeva-Gupta et al., 2011).

The monomeric G-protein family

The monomeric G-proteins have come to light in search for cancer-causing genes (oncogenes) in a group of related retroviruses, including the Harvey murine (H) virus (Harvey, 1964) and Kirsten sarcoma (K) virus (Kirsten et al., 1962). The transforming genes are fusions of the viral *gag* gene and one of the *ras* genes derived from rats and mice through which the virus had been passaged. A third member, N-Ras, was discovered much later as a transforming gene product in a neuroblastoma cell line (Shimizu et al., 1983). With ras sequences at hand, hundreds of monomeric G-proteins were identified by screening DNA libraries and their functions have necessarily been assigned subsequent to their discovery. To date, representative functions have been assigned for members of all the groups of monomeric G-proteins related proteins in vertebrates and also in other eukaryotic organisms such as yeast, flies, nematodes, and slime molds. Only a limited number of members of the monomeric G-proteins are known to be directly regulated by membrane signaling events. Ras members take central stage as they play a key role in growth factor signaling, and mutations in H-, K-, or N-Ras occur in a large percentage of human cancers and developmental disorders (Pylayeva-Gupta et al., 2011; Rauen, 2013; Cox and Der, 2010). Moreover, another member of the same subfamily, Rap1B, acts downstream of chemokine receptors and plays a role in activation of integrins in blood-borne cells (Shimonaka et al., 2003; Lim and Hotchin, 2012). Members of the Rho family, RhoA and Rac1 in particular, play a role in the same context; they enable chemokine-stimulated migration of cells by acting on the contractile (myosin II) and nucleation (WAVE/ARP) machinery of the actin cytoskeleton (Fleming et al., 2004). RhoA is also activated downstream of integrins and control the process of phagocytosis of murine macrophages (Wiedemann et al., 2006). We will return to these particular G-proteins extensively in later chapters.

GEFs and GAPs for monomeric G-proteins

The numerous monomeric G-proteins are regulated by a great variety of GEFs and GAPs, which we have briefly summarized in Table 2-4 (reviewed in Cherfils and Zeghouf, 2013). Monomeric G-proteins not necessarily have a unique concomitant GEF and GAP, in certain cases, for instance the Rho family, the GEFs and GAPs outnumber the G-proteins by threefold, in other cases, for instance Ras family, the G-proteins outnumber the GEFs and GAPs. The exchange reaction is initiated by the formation of a low-affinity complex between the GDP-bound G-protein and the GEF, which converts into a high-affinity nucleotide-free GTPase/GEF complex after dissociation of GDP. Binding of GTP eventually displaces the GEF, yielding the active form of the GTPase. Most GEFs project the switch-1 region away from the nucleotide-binding site by steric hindrance (thus widening the nucleotide-binding pocket). Second, all GEFs engage in extensive contacts with the switch-2 region. The formation of this large interface is probably important to stabilize the otherwise unstable nucleotide-free GTPase and protect it from unfolding (chaperone function). GEFs combine these two effects to facilitate the dissociation of GDP by different means but all causing a reduction in the affinity of Mg^{2+}/GDP for the binding pocket (Figure 2-26). As already mentioned, GAPs facilitate the transfer of the γ-phosphate to water (creating GDP + Pi) (Figure 2-27).

Despite sharing mechanistic hallmarks, both GEFs and GAPs reveal a stunning diversity in structure and composition (forming monomers, homodimers, or hetero-oligomers). GEFs and GAPs are big multidomain proteins (Figure 2-28) and this necessarily means that they are highly networked and subject to intramolecular regulation. The picture that emerges from numerous studies shows that GEFs and GAPs are auto-inhibited and require specific upstream signals to relieve the inhibitory constraint. Mechanisms of auto-inhibition and its release have now been described in detail for the SOS (RasGEF), EPAC (RapGEF), β2-chimerin (RhoGAP), and ArfGAP1 (Golgi ArfGAP). The GEFs and GAPs have probably roles beyond the control of the GTPase cycle for the reason that monomeric G-proteins do not have an intrinsic structural ability to sort between their many different effectors. Selectivity must be conveyed otherwise and an appealing view is that GEFs play a role in this process. They may channel G-proteins into binding to specific effectors by acting as scaffolds or by creating unique tripartite-binding sites (G-protein switch region + GEF + effector). Conversely, GAPs have means to select which active G-protein/effector complex has to be turned off. We end this section by making the point that the term monomeric G-protein is probably a misnomer because they appear to function in intricate complexes with GEFs, GAPs, and GDIs. These naturally regulate the duration of the active state but are also involved in the selection of effectors (Cherfils and Zeghouf, 2011, 2013).

The heterotrimeric G-protein family (Gα)

These G-proteins came to light as the missing link between the occupied beta-adrenergic receptor and the production of the second-messenger cAMP by adenylyl cyclase and they were named “transducers” (Brandt et al., 1983; Cerione et al., 1984). In their inactive state these Gα-proteins occur as complexes with Gβγ. The structural organization of the α, β, and γ-subunits is illustrated in Figure 2-30. The β-subunit, which has seven regions of β-sheets organized like the blades of a propeller

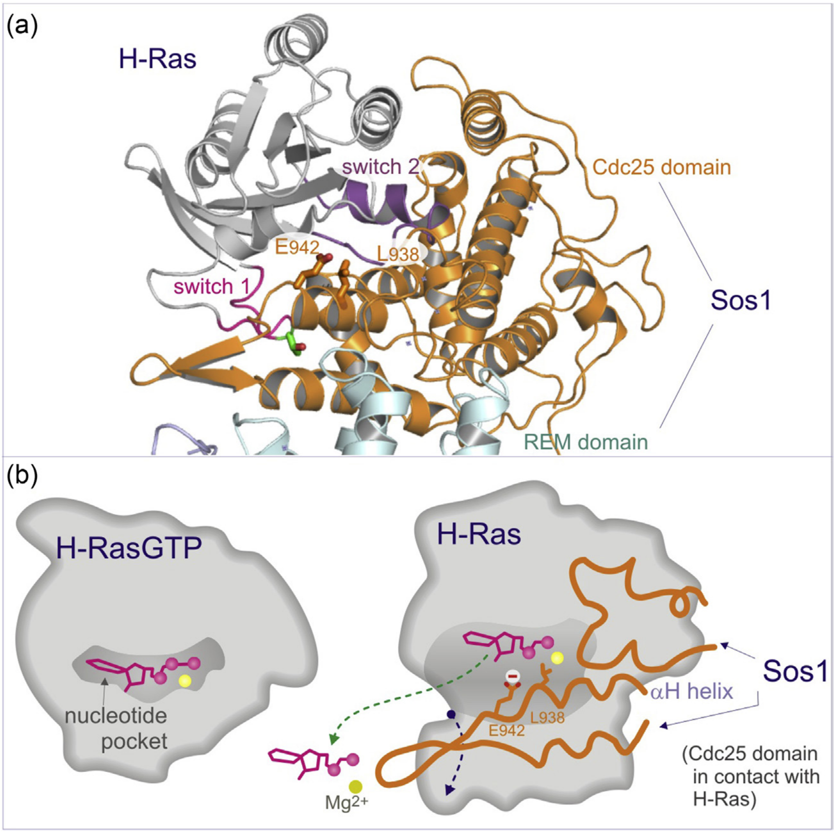


FIGURE 2-26 SOS-mediated hydrolysis of GTP in H-Ras. (a) SOS inserts a number of residues into the active site of H-Ras. It introduces a hydrophobic side chain (L938), which blocks magnesium binding, and an acidic side chain (E942), which overlaps the site where the α-phosphate of the nucleotide would otherwise be bound. As a result GDP binding to Ras is weakened. We have highlighted a residue from the switch-1 region, T35 shown in green, to illustrate how SOS pushes the switch-loop downward and, in doing so, opens the nucleotide-binding pocket. Pdb: 1bkd, (b) Semi-realistic representation of the changes that occur in the nucleotide-binding pocket upon binding of Sos1 to RasGDP. The αH-helix penetrates into the pocket and pushes the switch-1 region downward. This opens the pocket and allows GDP to escape. Moreover, E942 (Glu) causes electrostatic repulsion of α-phosphate and the nonpolar L938 (Leu) disrupts electrostatic coordination of Mg^{2+}. These two amino acids facilitate expulsion of Mg^{2+}/GDP, allowing GTP to enter the pocket.

(β-propellor), is tightly associated with the γ-chain and together they behave as a single entity. The α-subunit contains the nucleotide-binding site and its contacts with the β-subunit involve the switch-1 and –2 loops of the α-subunit as well as its N-terminal helix. The whole assembly is anchored to the membrane by lipid attachments to the α- and γ-subunits.

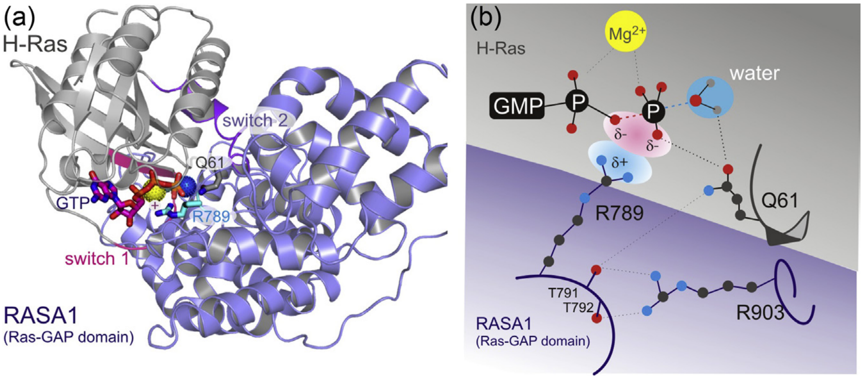
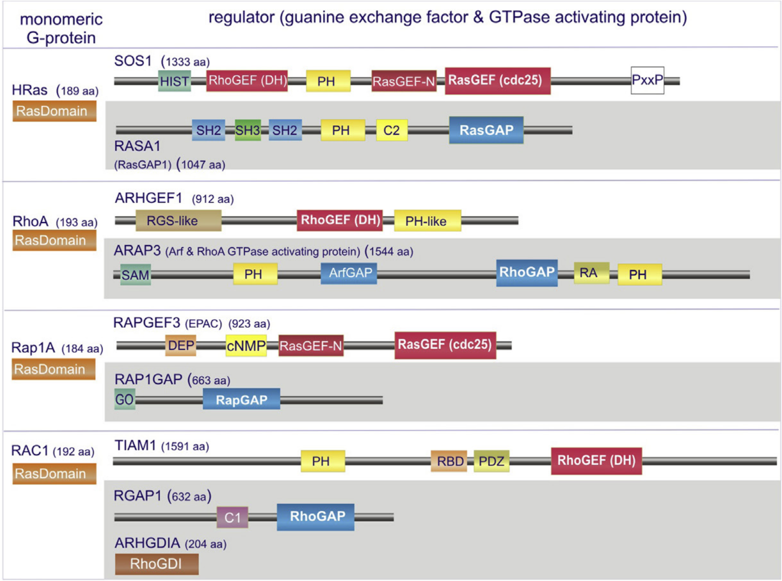


FIGURE 2-27 The action of RasGAP. (a) The GTPase-activating protein RASA1 (human RasGAP1) inserts an arginine (R789) into the nucleotide-binding pocket of H-Ras and this facilitates hydrolysis of GTP into GDP and Pi. Only the Ras-GAP domain is shown (195 residues of an otherwise 1047 aa protein), (b) Detail of the molecular structure around arginine-789. Its positive charge facilitates the phosphotransfer by reducing the energy required for reaching the intermediate state where phosphate is shared between the donor (guanine nucleotide) and the acceptor (water) (indicated by red and blue dotted lines). Gluamine61 of H-Ras coordinates the water molecule and the γ-phosphate, R903 coordinates the loop comprising R789. *Scheme adapted from Scheffzek et al. (1999). Pdb: 1wq1.*

The human genome contains 16 Gα-genes, giving rise to 21 different proteins (alternative splicing). Based on sequence similarity they are subdivided into four groups: Gαs, Gαi, Gαq/11, and Gα12. Typically, animal cells express 9 or 10 different Gα-subunits. Some, such as the GNAT1 and 2 (Gαt), GNAL (Gαolf), and GNAT3 (Gαgust) genes, are expressed only in single classes of sensory cells (photoreceptors, olfactory epithelial neurons, and taste receptors, respectively). Others are found in cells that share a common embryonic origin. Thus GNAO (Gαo) is expressed in cells derived from the neural crest and in endocrine tissues such as pancreatic β-cells (insulin production) and cells of the pituitary gland. GNAZ (Gαz) is found primarily in neurons, and GNA15 (Gα15/16) is expressed exclusively in cells of hematopoietic origin. Only GNAS (Gαs), GNAI2 (Gαi2), GNAQ (Gαq), and GNA12/13 (Gα12, Gα13) are universally expressed in animals cells. Figure 2-29 summarizes some of this information.

Gβγ-subunits

The human genome contains five Gβ genes, giving rise to six different proteins, and 12 Gγ genes which may create a large variety of dimers. In reality the Gβγ pairs are limited, for instance there is β1γ2 but not β2γ1. In general heterotrimeric G-proteins are defined by their



C1	(cystein rich-1),	connects with membrane (phospholipids)
C2	(cystein rich-2),	connects with membranes (phospholipids, sometimes Ca^{2+}-dependent)
cNMP	(cyclic nucleotide binding motif),	regulation by cAMP
DEP	(dishevelled-Egl10-pleckstrin),	connects with membrane (phospholipids) and with Gα/GPCR
GO	(GoLoco),	connects with heterotrimeric G-proteins in GDP-bound state
HIST	(histone)	
PH	(plekstrin homology),	connects with membrane (phosphoinositides PI3,4-P2, PI4,5-P3 or PI3,4,5-P3)
SAM	(sterile alpha motif),	connects with other proteins
SH2	(Src homology-2),	connects with phosphotyrosines (in specific amino-acid context)
SH3	(Src homology-3),	connects with proline-rich sequences (polyPro helix)
PDZ	(PDS-Dlg-ZO),	connects with lipids and proteins (common interaction motif)
RA	(Ras association domain),	connects with RasGTP (putative "effector" domain)
PxxP	(proline rich region)	connects with SH3 or WW domain
RasGEF (cdc25)	(guanine exchange factor)	connects with RasGDP and causes exchange of GDP for GTP
RasGEF-N		also known as REM (Ras exchanger motif), catalytically inactive, plays a structural role
RBD	(Ras binding domain),	connects with RasGTP
RGS-like	(regulator of G-protein signalling)	connects with GαGTP subunits, accelerates GTPase activity

FIGURE 2-28 Some examples of domain architecture of monomeric guanine exchange factors and guanine-activating proteins. Note that G-proteins are small single domain proteins, allowing little interactivity, other than binding certain effectors. Networking occurs through the multiple domains of their respective GEFs and GAPs. RhoGDI (guanine dissociation inhibitor) binds the geranylgeranyl lipid modification attached to cysteine-189 of RhoA. It prevents interaction with RhoGEF. Abbreviations: DH, Dbl Homology, Cdc25, cell division control protein-25 of yeast (contains a RasGEF domain).

Gα-subunit and not by their Gβγ, in other words, the diversity of these G-proteins is principally a function of their α-subunits (we speak of Gi, Gs, Go, etc.). Nevertheless, in spite of great difficulty in demonstrating specificity of Gβγ pairing in reconstitutions experiments, it has become clear that both the identity of Gβγ and the Gα-subunits play a

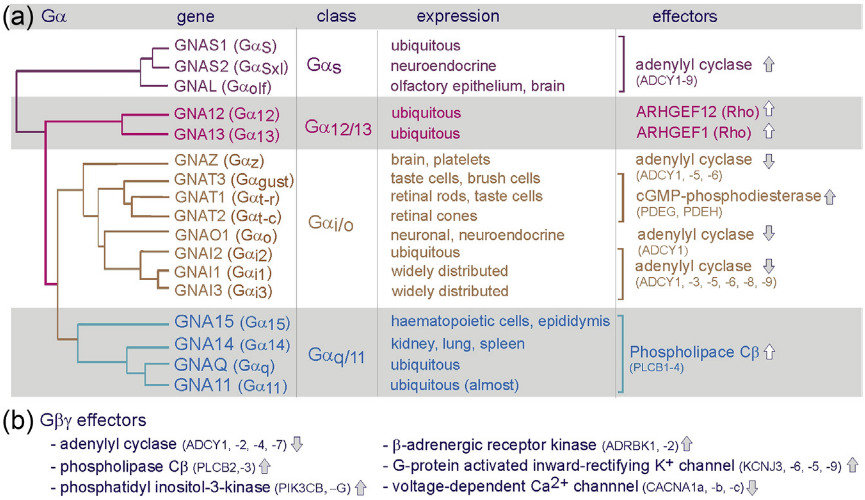


FIGURE 2-29 (a) The Gα family of G-proteins, their subdivision, expression patterns, and effectors. (b) Gβγ-subunits (identity not revealed) and their effectors.

role in the selective coupling to 7TM receptors. In the case where these receptors form dimers it is postulated that Gα and Gβγ bind to separate receptors but proof of this is still limited (Jastrzebska et al., 2010; Johnston and Siderovski, 2007).

The Gβγ-subunits qualify as signaling proteins as they are capable of interacting with effectors. The first indication that they transmit information was in connection with the process by which acetylcholine reduces cardiac rhythm (Logothis et al., 1987; Reuveny et al., 1994). Acetylcholine, released from the Vagus nerve, binds the muscarinic acetylcholine (M2) receptor both in the sinoatrial node (pacemaker) and on atria and this leads to activation of Gαiβ1γ2 (or Gαiβ2γ2, the choice of γ-subunits being free). Gαi inhibits adenyl cyclase, leading to a reduction in cAMP and this leads to a reduced phosphorylation of the RyR Ca^{2+} channel, hence a reduction in the contractile force of the atrial myocytes, but importantly, Gβ1γ1 binds inward-rectifying K^+ channels (KCNJ3, -6, -5, -9) and increases their conductivity. As a consequence of an increased outflow of K^+, the cell membrane hyperpolarizes and this reduces the cardiac rhythm (Morris and Malbon, 1999). Other Gβγ effectors are adenyl cyclase type II, -IV (stimulation), N-type voltage-gated Ca^{2+} channels (reduction of conductance), phospholipase A2 and phospholipase Cβ (stimulation), and phosphatidylinositol 3-kinase (stimulation) (Clapham and Neer, 1997) (see bottom section Figure 2-29). It is generally assumed that members of the Gαi/o-proteins are the major "source" of Gβγ-subunits.

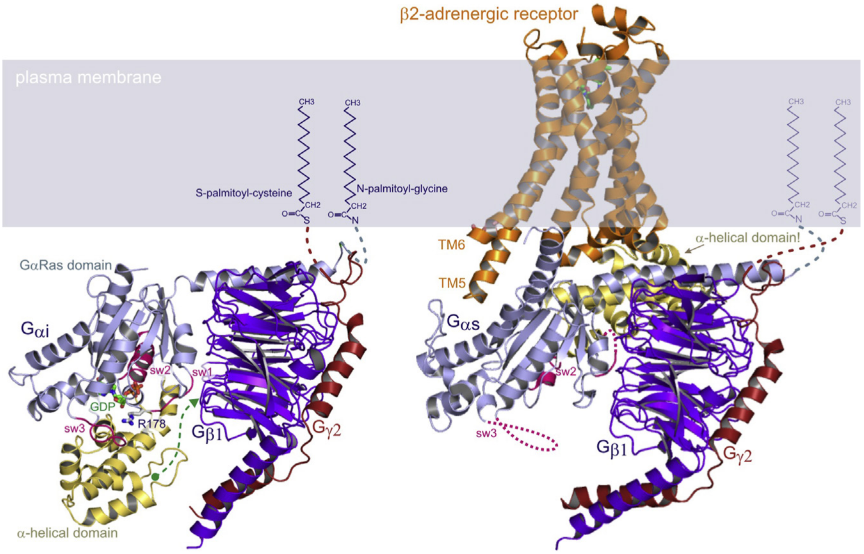


FIGURE 2-30 Left, heterotrimeric G-protein in GDP-bound state (rat Gαi1-β1-γ2, genes GNAI1-GNB1-GNG2). Right, heterotrimeric G-protein bound to the β2-adrenergic receptor (bos Taurus 16Gαs-β1-γ2 (GNAS, GNB1, GNG2). Note the presence of arginine-178 (involved in hydrolysis of GTP and lacking in monomeric G-proteins) and note that the AH domain is swung behind Gα and no longer aligns the nucleotide pocket. Both Gα and Gγ are attached to the membrane with an N-palmitoyl-glycine-2 and S-palmitoyl-cysteine-3 moiety, respectively Pdb: 1gg2, 3sn6.

GEFs and GAPs for heterotrimeric Gα-proteins

The human genome counts 800 GEFs for Gα-proteins, these are the 7TM (or GPCR) family of receptors (Fredriksson et al., 2003). Their mode of action varies from receptor to receptor. We highlight here the bovine β2-adrenergic receptor, where binding of adrenaline causes significant structural changes in the intracellular domains of transmembrane helices TM5 and TM6. Upon ligand binding, TM5 is extended by two helical turns whereas TM6 is moved outward by 14 Å (as measured at the level of Glu268) and these changes create a cavity in the intracellular side of the receptor into which the α5-helix of Gαs docks (Figure 2-30). The docking brings about changes in the nucleotide-binding pocket and weakens the interaction with GDP (it leads to perturbation of the β6–α5 loop, which coordinates the guanine ring, and of the β1–α1 loop, the Walker A motif that coordinates the β-phosphate in the GDP or GTP). The docking also causes a large displacement of alpha-helical domain relative to the GαsRas domain and as a consequence it no longer covers the nucleotide-binding site, allowing GDP to escape. It is not clear yet when exactly GDP is released during the formation of the receptor/Gα complex (Rasmussen et al., 2011).

The alpha-helical domain takes its original position once GDP has been replaced by GTP. Binding of GTP weakens the interaction with the receptor, and, remember, changes the switch-1, -2, and -3 loops (to which effectors now can bind). Other GEFs for heterotrimeric proteins are AGS1 (activator of G-protein signaling) and Ric-8, both are cytosolic proteins (Tall, 2013).

Heterotrimeric G-proteins have a higher intrinsic GTPase activity than monomeric but nevertheless their rate of hydrolysis is still much too slow to account for the transient nature of G-protein-mediated responses. The extreme example must be the visual transduction process that, of necessity, must be turned off rapidly. When the lights go off, we perceive darkness promptly. If Gαt (also named transducin1/2 or GNAT1/2) were able to persist in its active form, the illumination would appear to dim gradually as the hydrolysis of GTP proceeds (in the order of tens of seconds). It is now known that the effector of Gαt, cGMP phosphodiesterase, the enzyme that breaks down cGMP in response to light, acts as a GAP and rapidly terminates the response. As an exception, cGMP phosphodiesterase binds exclusively to the AH domain of Gαt and not to the GαRas domain (Liu and Northup, 1998). Other effectors with GAP activity are adenylate cyclase and phospholipase C (Waldo et al., 2010). One of the effector of Gα12 and Gα13 is a monomeric guanine exchange protein, RhoGEF, it couples a heterotrimeric G-protein signal to a monomeric G-protein (Rho) cascade (for domain structure return to Figure 2-28).

More generally, the GTPase activity of Gα is subject to control by a family of “regulators of G-protein signaling“ or RGS. The human genome counts 35, which are classified in groups from A–H, (Oldham and Hamm, 2008) and the majority binds to the GαRas domain (Figure 2-31) (Liu and Northup, 1998). They attenuate the signal by stimulating hydrolysis of GTP. RGS proteins appear to have no particular homologies, apart from their RGS homology domain (“RGS box” of ~120 amino acids). Their size ranges from 150 to 1400 residues (Siderovski and Willard, 2005). The bigger proteins posses multiple structural domains that allow intramolecular control and networking. For example, the above-mentioned RhoGEF is one of these multidomain proteins comprising a PDZ, RGS, DH, and PH domain. It binds and inactivates Gα12 (or Gα13) through its RGS domain. Somehow this interaction also relieves an intramolecular constraint, allowing expression of its guanine exchange activity of the DH domain. Three of the GPRs, members 10, 12, and 14, possess a GoLoco domain (Gαo-interacting polypeptide homologous to *Drosophila* locomotion defects proteins), which binds inactive Gαi and Gαo. The 14 amino acids that constitute the GoLoco domain stretches across both the GαRas and alpha-helical domains, thereby locking the protein in a GDP-bound state and preventing interaction with Gβγ. This in turn prevents interaction with the 7TM receptors and serves to prevent re-activation. These three members of RGS-box containing proteins act in a way similar to the GDI proteins described above (blocking re-activation of monomeric G-proteins).

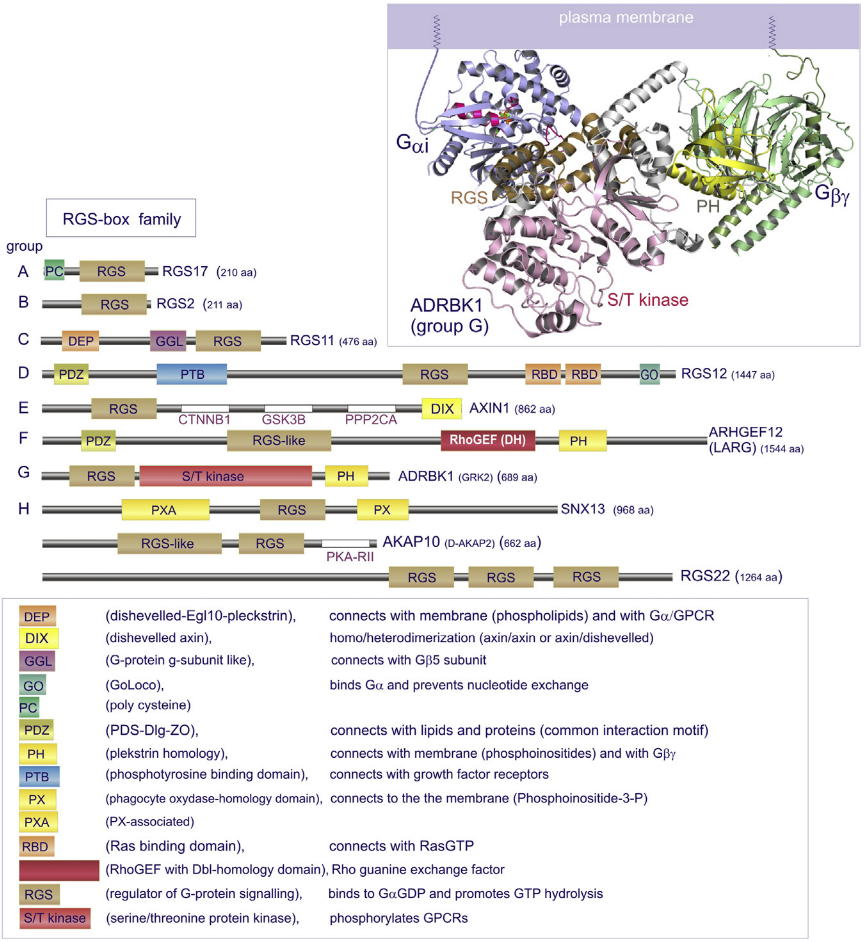

plasma membrane
Gαi
RGS
PH
Gβγ
ADRBK1
(group G)
S/T kinase
RGS-box family
group
A PC RGS RGS17 (210 aa)
B RGS RGS2 (211 aa)
C DEP GGL RGS RGS11 (476 aa)
D PDZ PTB RGS RBD RBD GO RGS12 (1447 aa)
E RGS CTNNB1 GSK3B PPP2CA DIX AXIN1 (862 aa)
F PDZ RGS-like RhoGEF (DH) PH ARHGEF12 (LARG) (1544 aa)
G RGS S/T kinase PH ADRBK1 (GRK2) (689 aa)
H PXA RGS PX SNX13 (968 aa)
RGS-like RGS PKA-RII AKAP10 (D-AKAP2) (662 aa)
RGS RGS RGS RGS22 (1264 aa)
DEP (dishevelled-Egl10-pleckstrin), connects with membrane (phospholipids) and with Gα/GPCR
DIX (dishevelled axin), homo/heterodimerization (axin/axin or axin/dishevelled)
GGL (G-protein g-subunit like), connects with Gβ5 subunit
GO (GoLoco), binds Gα and prevents nucleotide exchange
PC (poly cysteine)
PDZ (PDS-Dlg-ZO), connects with lipids and proteins (common interaction motif)
PH (plekstrin homology), connects with membrane (phosphoinositides) and with Gβγ
PTB (phosphotyrosine binding domain), connects with growth factor receptors
PX (phagocyte oxydase-homology domain), connects to the the membrane (Phosphoinositide-3-P)
PXA (PX-associated)
RBD (Ras binding domain), connects with RasGTP
(RhoGEF with Dbl-homology domain), Rho guanine exchange factor
RGS (regulator of G-protein signalling), binds to GαGDP and promotes GTP hydrolysis
S/T kinase (serine/threonine protein kinase), phosphorylates GPCRs

FIGURE 2-31 Domain architecture of RGS box-containing proteins. numerous domains connect these proteins to various intracellular sites. The insert shows the β-adrenergic receptor kinase (ADRBK1) which is bound to GαGTP with its RGS domain. The three switch regions of Gαι are shown in pink. the PH domain of ADRBK1 is bound to Gβγ. The serine/threonine protein kinase-domain situates between the RGS and PH domains. The helices in light gray connect the different domains which each other. Pdb: 2bjc.

G-protein-coupled receptor kinases and receptor desensitization

One of the subfamilies of the GRS-box containing proteins (group G in figure 2-31) also possesses a serine/threonine kinase domain. This is capable of phosphorylating 7TM receptors and they therefore qualify as G-protein-coupled receptor kinases or GRKs. The human genome contains seven such kinases. The RGS-box serves to dock the protein kinase to an active Gα and so brings the kinase near the receptor. The β2-adrenergic

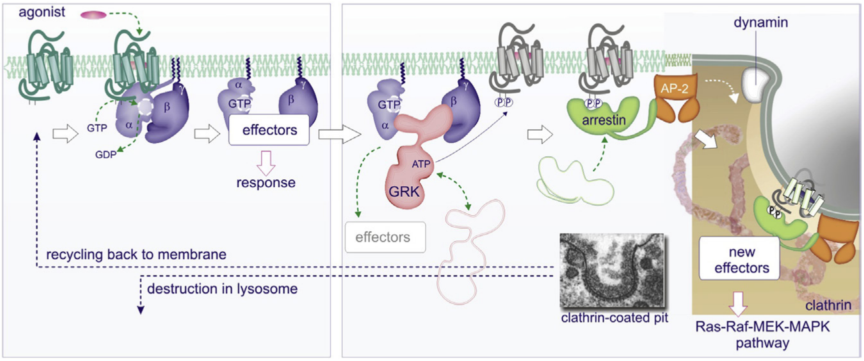


FIGURE 2-32 Receptor desensitization through phosphorylation. The ligand-bound GPCR activates a heterotrimeric G-protein through the exchange of GDP for GTP. This causes binding and activation of effectors, leading to the production of second messengers and a cellular response. The activated G-protein also binds the G-protein-coupled protein kinase (GRK) and in doing so they get phosphorylated onto their cytosolic segment. This prevents further interaction with effectors and the receptor is said to be "desensitized" (production of second messenger seizes). Arrestin binds the phosphorylated receptor and it serves as a platform for yet other effectors such as those constituting the MAPK pathway. Arrestin also facilitates receptor endocytosis, via clathrin-coated pits. From here the receptor can recycle back to the cell membrane, after having lost its ligand, or progress toward the destructive lysosome.

receptor has four serine residues accessible to GRKs but these are not necessarily all phosphorylated. The extent of phosphorylation depends on the GRK that is recruited to the membrane (Nobles et al., 2011). As a consequence of phosphorylation, the receptor binds β-arrestin thus preventing further interaction with G-proteins. In the sequence of events going from β-adrenergic receptor to stimulation of adenylcyclase, the phosphorylation leads to an attenuation of the production of cAMP, despite the continuous presence of adrenaline, and the receptor is said to be "desensitized." This however is not the whole story; β-arrestin also acts as a docking protein for other effectors and from here new signaling pathways are initiated. One of these is the Ras-ERK pathway, normally employed by growth factor receptors (Shenoy et al., 2006). β-arrestin also causes endocytosis of the receptor and this may lead to dissociation of the ligand and possibly receptor degradation in late endosomes (Figure 2-32).

GRKs do not have the exclusive right to phosphorylate 7TM receptors. One other protein kinase, activated by the second-messenger cAMP, and termed PKA, also phosphorylate these receptors (but at different residues). We return to PKA later in this chapter. Apparently PKA does not discriminate between receptors and can cause desensitization of a wide range, not just β2-adrenergic receptors. This phenomenon is called "heterologous desensitization."

HETEROTRIMERIC G-PROTEINS ARE TARGETS OF TOXINS

Vibrio cholerae, causing severe diarrhea, and *Bordetella pertussis*, causing whooping cough, produce toxins that affect heterotrimeric G-proteins. Cholera and pertussis toxin are enzymes that modify their target proteins by covalent attachment of the ADP-ribosyl moiety of NAD^+ (Gill and Meren, 1978; Cassel and Pfeuffer, 1987). Cholera toxin transfers ADP-ribose to Gαs forming a covalent attachment with an arginine residue (R201) situated in the close vicinity of γ-phosphate of the bound GTP. This modification prevents the GTPase activity and so causes the α-subunit to remain persistently activated and hence persistently activates adenyl cyclase. Pertussis toxin transfers ADP-ribose to Gαi at a cysteine situated just four residues from its C-terminus. ADP-ribosylation by pertussis toxin has the effect of interrupting the communication between the receptor and Gαi, freezing it in its GDP-bound state. In this way it prevents the negative signal from inhibitory receptors with the same consequence as cholera toxin, namely persistent activation of adenylyl cyclase and elevated levels of cAMP. With the exception of Gαz, all members of the Gi/Go group of α-subunits serve as substrates for pertussis toxin. In most cells and tissues, Gαs is the unique substrate for cholera toxin. Elongation factors are also a target for pathogen toxins. *Corynebacterium diphtheriae* produces the diphtheria toxin that alters protein function in the host by inactivating elongation factor (eEF-2). This causes pharyngitis and pseudomembranous inflammation in the throat. As reagents, cholera and pertussis toxins have been instrumental in assigning particular receptors to particular G-proteins in signaling pathways that is, them being sensitive or not to one of the toxins.

Web resources

- HUGO Gene names:
  - “regulator of G-protein signaling” (RGS) http://www.genenames.org/genefamilies/RGS.
  - Arf http://www.genenames.org/genefamilies/ARF http://www.genenames.org/genefamilies/ARFGAP.
  - Rab http://www.genenames.org/genefamilies/RAB.
  - Rho http://www.genenames.org/genefamilies/ARHGAP http://www.genenames.org/genefamilies/ARHGEF.

A BRIEF DEFINITION OF EFFECTORS

Historically speaking "effectors" were defined as enzymes that act directly downstream of heterotrimeric G-proteins. Within this definition, two classical effectors stand out and they are adenylyl cyclase and phospholipase C. Both generate second messengers, the first cAMP and the second a membrane-bound diacylglycerol (DAG) and a cytosolic inositol 1,4,5-trisphosphate (IP3) (Michell, 1975; Sutherland et al., 1962). But if we consider that effectors are proteins that bring about an effect, a cellular response, than, in the context of signaling events, they occur at many levels. Take the adrenaline pathway. Adenylyl cyclase is an effector because it brings about the production of a second messenger as a consequence of activation by a heterotrimeric G-protein. But then PKA brings about an effect in response to the rise in cAMP, it activates phosphorylase kinase, and phosphorylase kinase effectuates the activation of glycogen phosphorylase and this enzyme assures the production of glucose-1-phosphate. The acetylcholine-mediated rise in intracellular free Ca^{2+} causes the activation of numerous effectors, these are the proteins that either directly bind Ca^{2+} or interact with a Ca^{2+}-binding subunit such as calmodulin. One of the effectors is Troponin (calmodulin-like) associated with tropomyosin and when these are displaced on the actin filament, as a consequence of Ca^{2+} binding, they allow the interaction between actin and myosin. Myosin can be considered an effector as it brings about contraction. Phosphorylase kinase and adenylyl cyclase are also complexed with calmodulin and these too are Ca^{2+} effectors (their activity being boosted) (return to Figure 2-18). Effector proteins are plentifold.

FOCUS ON PROTEIN PHOSPHORYLATION

The discovery of phosphoproteins

Phosphoproteins were discovered when proteins were discovered. This dates back to 1835, when Gerrit Mulder, working as a physician in Rotterdam and Utrecht during the day and as a chemist in the night, analyzed the chemical composition of egg white. He concluded that it consists of a base ("wortelstof"), comprising carbon, hydrogen, oxygen, and nitrogen, to which is added a little phosphorus and sulfur. Mulder went on to show that substances of identical composition, the so-called "eiwitagtige ligchamen," were present in various tissues, both in animals and in plants. In his own words: "If one thing is certain, I am the first to show that the meat is present in the bread (wheat) and the cheese in the grass." Mulder proposed that living material is composed of "wortelstof" but with varying amounts of phosphorus and sulfur. At present, we know that the differences between proteins are due to their variable amino-acid composition. Due to the abundant presence of "wortelstof" in all living tissue Jöns Jacob Berzelius (Stockholm, Sweden) suggested in a letter to Mulder, in 1838, to name it "protein," from the Greek "protos" meaning "first." Thus, the current concept of protein was established: "wortelstof" + phosphorus + sulfur = protein. Mulder's ideas were adopted by the famous German chemist Justus von Liebig (Munich, Germany) who wrote in his book "Thierchemie" in 1842 that "living nature is composed of carbohydrates, lipids and proteins." A new branch of chemistry, termed biochemistry, was born.

At the start of the twentieth century it became apparent that certain egg and milk proteins, vittelin and casein, respectively, contain unusual large amounts of serine phosphoric acid (Levene and Alsberg, 1906; Lipmann et al., 1926; Lipmann and Levene, 1932). In 1954 it was shown that mitochondria contain an enzyme capable of incorporating radioactive inorganic phosphate into the protein fraction "at a rather rapid rate" (Burnett and Kennedy, 1954). Of the many substrates tested, alpha-casein came out as the best and the corresponding mitochondrial kinase was named casein kinase (CK1). We now know that casein kinase is not really involved in casein phosphorylation but serves as a key protein kinase in a number of signaling pathways. Until 1955 the general idea prevailed that phosphoamino acids serve as a storage reservoir for phosphorus, and this is true for vittelin and casein (Perlmann, 1955).

PHOSPHORYL GROUP, PHOSPHORIC ACID, PHOSPHATE, AND INORGANIC AND ORGANIC PHOSPHATES

Phosphoryl groups are derivatives of phosphoric acid (H_3PO_4). Phosphorus has three unpaired electrons in 3p orbital and following the octet rule it would interact with only three oxygens. However, it positions at the third row of the periodic table, offering an extra 3D orbital available for bonding. It therefore can break the "octet rule" and offer a total of five bonds in a tetrahydral geometry. The fully deprotonated conjugate base of phosphoric acid is a phosphate ion, also known as inorganic phosphate (Pi). When two phosphates are linked by an anhydride bond the ion is called inorganic pyrophosphate (PPi). When a phosphate is bound to an organic molecule, an amino acid, glucose, or nucleoside, the linkage is referred to as phosphate ester and the whole species is called an organic phosphate. To distinguish from a free phosphate we write P, hence, P-serine, ATP, or G6P. Once hydrolyzed, the liberated phosphate

continued

PHOSPHORYL GROUP, PHOSPHORIC ACID, PHOSPHATE, AND INORGANIC AND ORGANIC PHOSPHATES *(cont'd)*

becomes inorganic and we write ATP→ADP+Pi. Phosphoric acid can donate three protons with p*K*a values of 2.1, 7.2, and 12.3, respectively. In a physiological environment, pH 7.3, two of the three protons of phosphoric acid will be liberated $\left(HPO_4^{2-} + 2H^+\right)$. Organic phosphates are predominantly deprotonated and by convention are drawn in their fully deprotonated state $\left(R\text{-}PO_4^{3-} + 3H^+\right)$. The term "phosphoryl group" is a general way to refer to all of the phosphate-based groups mentioned above. The transfer of the phosphoryl group is called phosphorylation (or sometimes phosphotransfer), a process catalyzed by "kinases."

Phosphorylation determines the activity of glycogen Phosphorylase

A change in thinking about the purpose of phospho-proteins came about when it was shown, by Krebs and Fischer in 1956, that the difference in basal activity of purified glycogen phosphorylase, named phosphorylase *b* in its low-active state and phosphorylase *a* when recovered with high basal activity, could be explained by the addition or removal of a single phosphate (Krebs and Fischer, 1956). This discovery provided first evidence that phosphorylation could alter enzyme activity (return to Figure 2-16). Soon it became apparent that numerous proteins are subject to phosphorylation, for instance glycogen synthase and phosphorylase kinase, and that this too plays a role in regulation of their activity. In 1988, a highly detailed analysis (by X-ray crystallography) revealed that phosphoserine-14 of glycogen phosphorylase interacts with a number of arginines at the N- and C-terminal regions, and these lead to strengthened interactions between subunits (the enzyme exists as a symmetrical dimer) and alter the binding sites for allosteric effectors and substrate (Sprang et al., 1988). The consequence of phosphorylation is best visualized by a change in positioning of a nine amino-acid loop near the catalytic site. Its position shifts from a tense state, in which it hinders catalytic activity, to a relaxed state, which permits catalytic activity (return to Figure 2-16). Numerous studies have followed about how phosphorylation controls protein structure and function, in particular with respect to the enzyme that controls the phosphorylation reaction (protein kinases too are subject to control by phosphorylation) (Johnson and Lewis, 2001).

PROTEIN PHOSPHORYLATION IN AN ERA WHERE ENZYMES WERE THOUGHT TO BE REGULATED BY METABOLIC INTERMEDIATES (ALLOSTERIC FACTORS)

As the pace of research on protein phosphorylation quickened during the 1970s, investigators began to question why it was ‘necessary’ to have two broad systems for controlling enzyme activity, i.e., allosteric regulation through the reversible binding of ligands (metabolic intermediates) and reversible covalent modification such as phosphorylation and dephosphorylation. The question was particularly pertinent in reference to substrates such as phosphorylase and glycogen synthase, since each of them was known to be subject to regulation by positive and negative allosteric effectors. Moreover, it was clear that allosteric and covalent regulation probably worked through similar conformational changes in both of these proteins. However, a basic difference between these two types of regulation soon became apparent. While allosteric control generally reflects intracellular conditions (energy charge or redox potential of the cell, fluctuations in metabolic intermediates, etc.), covalent regulation responds mainly to extracellular signals (Fischer and Krebs, 1989).

Driving the phosphorylation reaction, the role of triphosphate nucleotides

Kinases are the enzymes that transfer phosphate onto protein (and other cellular components). We return to them later. Of a long list of potential phosphate donors, the transfer reaction in eukaryotic cells is mainly driven by triphosphate nucleotides. Of these, ATP is the most popular one (Table 2-5) although for a few protein kinases, GTP appears to be an alternative source (PKCδ, CaMK, CKII, Mst-3 kinase, and bacterial aminoglycoside kinases). We note that bacteria possess a protein kinase, named E1-kinase, which phosphorylates a histidine residue with the use of phosphoenolpyruvate (PEP). It is unclear to us why ATP has turned out to be the favorite nucleotide for phosphate transfer, whereas GTP has been chosen for nucleotide exchange (G-proteins). With ATP, the free energy of the hydrolysis reaction is large (~30 kJ/mol) and this drives the transfer reaction. About half of the energy is used in making the phosphate transfer, the other half is conserved in the new phosphate-protein bond (so as to enable a subsequent dephosphorylation reaction) and some must be transformed into heat. Do not

TABLE 2-5 Different sources of phosphate, potentially amenable for a transfer reaction

Potential phosphate donors	
Source	**Free energy of hydrolysis (kJ/mol)**
Phosphoenolpyruvate (PEP)	61.9
1,3-bisphosphoglycerate	49.4
Creatine phosphate	43.1
ATP, TTP, UTP, GTP, CTP	30.5
ADP, TDP, UDP, GDP, CDP	30.0
Glucose-1-phosphate	20.9
Pyrophosphate (PPi)	19.3
Glucose-6-phosphate	13.8
Glycerol-3-phosphate	9.2
Phosphate donors in cellular processes	
ATP	Nearly all protein kinases (500 or so)
GTP	CKII, CaMK, PKCδ
CTP	Dolichol (isoprenoid) kinase
PEP	EI kinase (autophosphorylation on histidine in bacteria)
PPi	Phosphofructokinase (in prokaryotes and plants)
Pi	Glycogen phosphorylase
NTP	Nucleoside kinases (dTK, dCK, dGK)

underestimate the importance of phosphate hydrolysis and phosphate transfer (ATP→ADP + Pi (or Pi-substrate)) in your daily life because humans turnover roughly their bodyweight each day! This of course also includes the use of ATP for the functioning of motor proteins (such as myosin, dynein, kinesin, and DNA helicases), of membrane pumps (Na^+/K^+-ATPase, Ca^{2+}-ATPase, H^+/K^+-ATPase, etc.), of actin (polymerization process), and of nucleoside kinases involved in the production of other nucleotides such as IMP, GTP, UTP, CTP, and TTP (and their deoxy-versions necessary for the replication of DNA).

NUCLEOPHILIC SUBSTITUTIONS TO PHOSPHORUS

In signal transduction we distinguish three types of phosphoryl transfer: (1) from a triphosphonucleotide to water (ATPase, GTPase, and ATP- and GTP-cyclases); (2) from a phosphonucleotide to an alcohol (kinase); and (3) from an organic phosphate to water (phosphatase). In chemical terms this is referred to as nucleophilic substitutions to phosphorus. For all situations the reaction can be described as: nucleophile$^-$ (receiver) + PO_3–X (donor) → nucleophile-PO_3 + X^-. The reaction mechanism, the formation of intermediate states in which the nucleophile and donor transiently "share" the phosphoryl group, may vary according to the catalysts involved. What catalysts do is creating a scaffold that reduces the energy required for reaching the intermediate state (or states) of the reaction. For a better comprehension of nucleophilic substitutions to phosphorus suggest the following articles and their references: (Allen and Dunaway-Mariano, 2004; Mildvan, 1997).

ATP AS A SOURCE OF ENERGY OR JUST A SOURCE OF PHOSPHATE?

ATP hydrolysis is considered as the final link between the energy derived from food (or sunlight) and cellular activities necessary to maintain life (such as muscle contraction, the establishment of ion gradients across membranes, and biosynthetic processes). ATP is therefore often considered as the source of energy to drive these processes. The majority of students interpret this as if the hydrolysis of ATP provides the energy to bring proteins in an active state (as in "waking up the enzyme and make it do things"). The "energy" approach to signaling events (or other ATP-requiring activities) is not necessarily wrong but it obscures the shape-change message we have mentioned previously. To our understanding, conformational changes seem to be at the basis of all cellular processes. Within a limited range of free energy these shape changes can occur spontaneously but for larger changes it is necessary to modify electrostatic interactions and hydrogen bonds through changes in protein composition or through interaction with allosteric regulators. Indeed, the transfer of phosphate is enabled by the high energy state of ATP, but the structural and functional consequences appear to be independent of the energy liberated by the breakage of the anhydride bond. That is why in many instances allosteric factors

continued

ATP AS A SOURCE OF ENERGY OR JUST A SOURCE OF PHOSPHATE? *(cont'd)*

also can bring about the same changes without carrying the "energy" of ATP. The same line of thinking applies for cycles of guanine-nucleotide exchange and subsequent hydrolysis, case of G-proteins, or for cycles of adenosine nucleotide exchange and subsequent hydrolysis (case of actin and motor proteins). Remember, with respect to nucleotide exchange and hydrolysis, shape changes occur without a covalent attachment of phosphate to the protein.

ENERGY

The cheapest energy is that which is not consumed, says the European directive on energy efficiency. It is true that for each of us, energy is primarily an electricity, gas, or fuel bill and saving energy translates in reducing the amount of the bill. But before being a financial issue, energy is primarily a physical concept which quantifies the ability of a system to perform transformations. Mobility, heating, cooking food, or making an object all requires energy. The more the transformation is important, the more energy is required. Ever since the handling of fire, mankind has domesticated many forms of energy, which has enabled a remarkable increase of material production. Energy has a fundamental property: it can neither be created nor be destroyed: it keeps. In this respect it is misleading to speak of production or consumption of energy. However, energy can be transformed. Ultimately, a journey made by a car in a closed circuit can be summed up as the transformation of fuel into heat (when you leave and start at the same place). Thus, the energy is degraded as it passes from a chemical form (petrol) into a mechanical (movement) and finally into heat and $CO_2 + H_2O$! Signaling pathways involve a lot of transformations and therefore require an input of energy (extract from (Lehoucq, 2012)).

Consequences of phosphorylation

Phosphate is very useful in bringing about shape changes (allostery) because: (1) it adds negative charges to a protein allowing for a change in electrostatic interactions that lead to conformational changes (interactions with positively charged lysine, arginine, and histidine); (2) it has the ability to form three or more hydrogen bonds and the tetrahedral geometry makes these hydrogen bonds highly directional allowing for strong interactions with hydrogen-bond donors (interactions with polar uncharged asparagines, glutamine, serine, and threonine); (3) it forms a stable but not too stable bond, which makes the modification reversible; and (4) enzymes control its addition (kinases) and removal (phosphatases), which makes the process highly "regulatable" (controllable).

In the example of glycogen phosphorylase, phosphorylation modifies protein structure and renders the enzyme more active. Phosphoserine-14 interacts with three adjacent arginines and this has long range effects on subunit binding and the positioning of amino acids at the catalytic site. Another example, where multiple phosphorylations cause an important change in conformation is the insulin receptor. It should be noticed that phosphorylation does not always activate, it can also inhibit enzymes. The serine/threonine protein kinase Cdc2 is inhibited by phosphorylation of threonine-14 and tyrosine-15. Moreover, phosphorylation does not always lead to shape changes. Phosphorylation of intrinsically unstructured proteins (proteins that lack 3D structure), or unstructured segments of proteins, serve to create docking sites for other proteins. The EGF receptor recruits enzymes to the membrane by phosphotyrosine residues in the unstructured C-terminal segment. In this particular situation, phosphorylation leads to the recruitment of signaling complexes. However in certain instances the phosphorylated unstructured segment is recognized by a domain within the protein and this leads to changes in conformation as well as activity. The tyrosine kinase Src is regulated this way. Its SH2 domain binds to a phosphotyrosine situated in the unstructured C-terminal tail and this leads to a deformation of its catalytic subunit. The intramolecular binding renders the kinase inactive much in the same way as insertion of myristate into the catalytic subunit of c-Abl (return to Figure 2-21(b)). As a last example, phosphorylation of serine-9 of the unstructured N-terminal segment of the glycogen-synthase kinase, GSK3β, creates a pseudosubstrate that nicely fits the catalytic cleft. By lacking a phosphorylation site, the N-terminal segment acts as a "pseudo-substrate," an antagonist that blocks entry of substrates. Examples of consequences of protein phosphorylation are shown in Figure 2-33.

PROTEIN KINASES CATALYZE THE PHOSPHATE TRANSFER

Protein kinases structure and function

ATP is a kinetically stable but thermodynamically unstable nucleotide. What this means is that ATP would like to get rid of its third phosphate

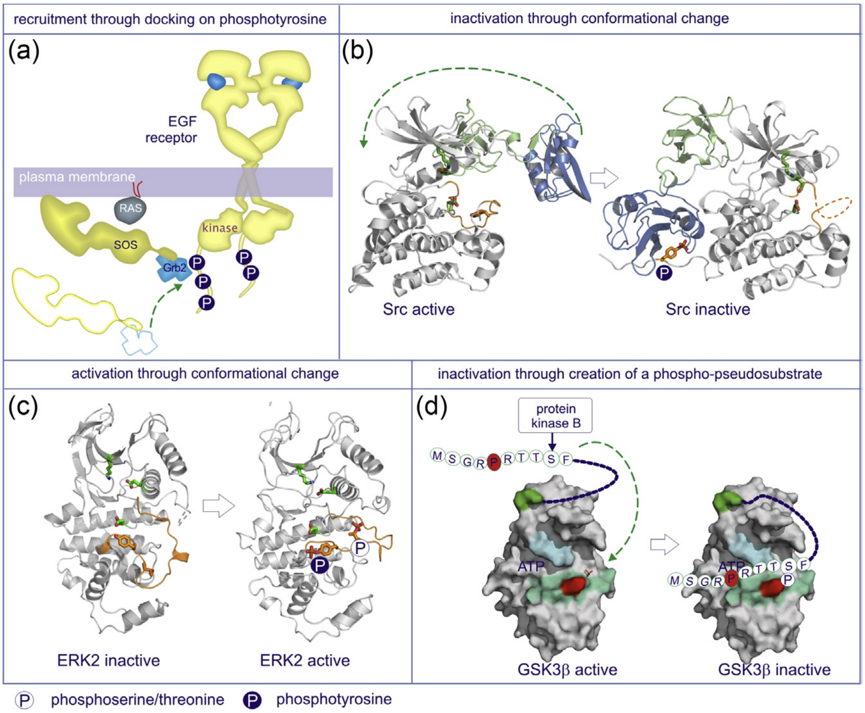

recruitment through docking on phosphotyrosine
inactivation through conformational change
(a)
EGF receptor
plasma membrane
RAS
kinase
SOS
Grb2
P
(b)
Src active
Src inactive
P
activation through conformational change
inactivation through creation of a phospho-pseudosubstrate
(c)
ERK2 inactive
ERK2 active
P
(d)
protein kinase B
M S G R P R T T S F
ATP
GSK3β active
GSK3β inactive
P phosphoserine/threonine
P phosphotyrosine

FIGURE 2-33 Consequences of protein phosphorylation. (a) Phosphorylation on C-terminal tyrosine residues in the intracellular segment of the EGF receptor causes binding of Grb2 through its SH2 phosphotyrosine-binding domain. Grb2 in turn is attached to SOS, a nucleotide exchange factor for RAS. This way, signaling complexes are formed around the activated growth factor receptor, (b) Phosphorylation of the C-terminal tyrosine of the protein kinase Src leads to an intramolecular attachment of its SH2 domain (in blue). This brings about important conformational changes that cause inactivation of its catalytic activity, (c) Phosphorylation of ERK2 on both a tyrosine and a serine residue leads to conformational changes that render the kinase catalytically competent. Note the profound changes in the so-called "activation segment" (colored in orange), (d) Phosphorylation of a phosphoserine residue in the N-terminal of the protein kinase GSK3β creates a phospho-pseudo-substrate. The phosphopeptide now binds the catalytic cleft of the protein kinase but unlike real substrates it lacks a serine or threonine four positions upward. As the image shows, this position is occupied by a proline (in red) and as a consequence the kinase remains stuck with its own N-terminal tail. Pdb: 1fmk, 1y57 (Src); 2erk, 3qyw (ERK2); 1uv5 (GSK3β).

(in the γ-position), and to a lesser extent its second phosphate (β-position) because that would be energetically favorable (the end products are more stable). Unfortunately in the absence of a suitable catalytic scaffold the transfer of phosphoryl group is prevented (because of electrostatic repulsion of water). That is why ATP keeps for years in a fridge (being "kinetically stable").

Protein kinases act as a catalytic scaffold by positioning a nucleophile (an atom in the substrate that donates an electron, which in this context are $–O^{-}$, $–S^{-}$, or $=N^{-}$), in close vicinity of the electrophilic γ-phosphorus of ATP (which attracts electrons). The nucleophile then donates an electron to the phosphorus, which ultimately leads to a break of the anhydride bond and finishes with PO_3^{3-} being attached to the substrate (phosphotransfer). The reaction only occurs if the distances between the atoms (nucleophile, phosphorus of phosphate, and an OH of the catalytic amino acid) do not exceed 4.5 Å. As mentioned previously, the reaction is exorgenic; it liberates energy because ADP is more stable than ATP.

In order to succeed phosphate transfer, protein kinases must fulfill a number of requirements: (1) they must bind ATP; (2) they must coordinate two magnesium ions (Mg^{2+}) to make phosphorus even more electrophilic; (3) they must reduce electrostatic repulsion of the oxygen groups; (4) they must bind substrate; (5) they must bring the phosphorus and the nucleophilic atom of the substrate within a range of 4.5 Å; and (6) they must render the nucleophile of the substrate still more nucleophilic (see Figure 2-34). Moreover, once the substrate is phosphorylated, ADP has to be removed and replaced by ATP and the substrate has to detach in order to allow access for a new substrate. Catalytically competent ("active") protein kinases are able to do all this because of a precise positioning of a number of key amino acid residues.

Protein kinases, or more precisely the kinase domain of protein kinases, are typically composed of a catalytic cleft enclosed by an N-terminal and C-terminal lobe. The N-terminal lobe coordinates ATP which is inserted in a pocket that is covered by a glycine-rich "P-loop." The C-terminal lobe also coordinates ATP, in particular the γ-phosphate, but acts as the main site for substrate binding and it carries the catalytic residue, aspartate (D), in a highly conserved -RDL- motif. In numerous instances the C-lobe controls the orientation of the αC-helix in the N-terminal domain. The role of the "catalytic" aspartate is to render the OH-group of the substrate more nucleophile (by removing a proton (H^{+})). The position of conserved residues, those that characterize typical eukaryotic protein kinases (ePK) are shown in Figure 2-35. With a few exceptions, the primary amino acid sequence (the native unmodified protein) gives rise to an inactive protein kinase. Activation requires one or more posttranslational modifications in order to obtain the above-described catalytically competent configuration. How protein kinases obtain their active state varies enormously (Jura et al., 2012) but once they are catalytically competent they resemble each other a lot.

ANHYDRIDE

Anhydride means "loss of water" as it is used to create an acid ($COO^{-} + H^{+}$) when breaking up the bond.

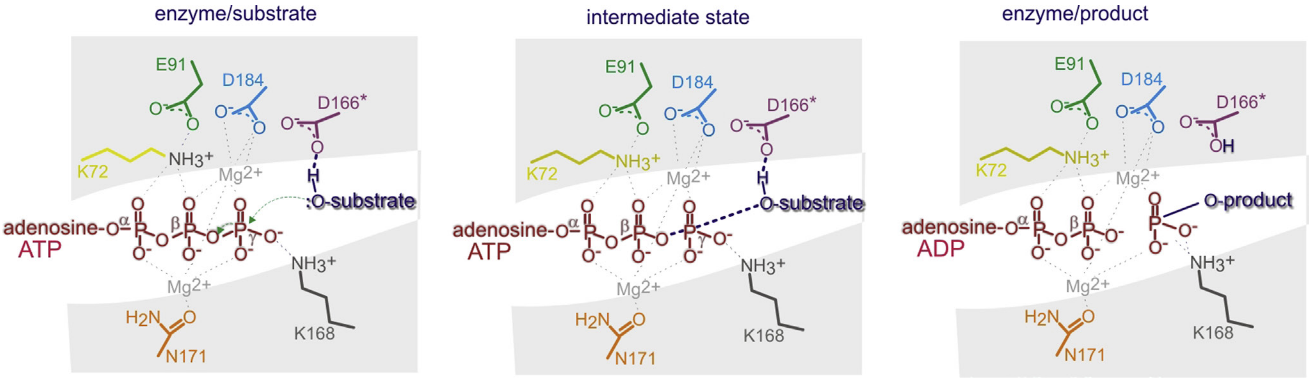


FIGURE 2-34 Schematic representation of protein kinase catalytic mechanism. The reaction proceeds from the enzyme/substrate complex through the transition state (center) to the enzyme/product complex. Residue numbers are for protein kinase A (as shown in Figure 2-35(a)). *Image adapted from Endicott et al. (2012).*

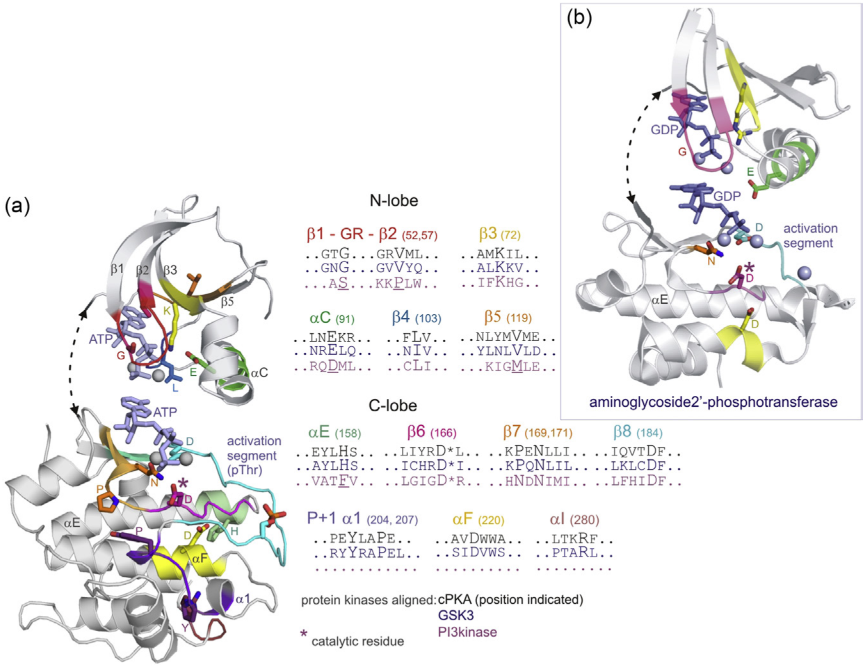


FIGURE 2-35 Conserved sequences and structural characteristics of pro- and eukaryotic protein kinases. (a) Alignment of conserved motifs on a structural characters matrix of two typical eukaryotic protein kinase, cPKA (mouse) and GSK3β (human) with one atypical PI3K (pig). The corresponding regions in the structure of cPKA have similar colors. The numbers refer to the position of the highly conserved amino acids in cPKA. The N-lobe has been separated from the C-lobe (dashed arrow in hinge region). Note that PI3K is quite distinct and yet considered to belong to the superfamily of protein kinases (albeit it is an atypical member). The α and β refer to numbered α-helices and β-sheets that make up the structure. GR, glycine-rich loop, involved in the binding of ATP. P+1 (substrate-binding segment) is a term used for tyrosine protein-kinases to indicate the amino acid that interacts with residues in the substrate one position (and beyond) from the phosphorylated residue. Note the absence of the α1, αF, and αI sections of the atypical PI3-kinase, sections that play an important role in substrate selection, (b) Structural characteristics of aminoglycoside 2″-phosphotransferase IIIa. There is a hinge (in black), an N-, and C-lobe. The glycine-rich loop GxxxG is present, arginine (N171) is present (N201), and lysine K72 seems to be replaced by arginine (R44 in β3), catalytic aspartate (D166) is in the sequence HGDL at position (D194), the activation segment is absent but the conserved aspartate (D216) in the DFG motif (in β8), which normally precedes it, is present.

The kinase-fold, but not necessarily the overall amino acid sequence of the kinase domain, is highly conserved among species. To illustrate this, whereas bacterial aminoglycoside kinases only have 10–17% sequence identity with ePK, they share a nearly identical kinase-fold (Figure 2-36) (Hon et al., 1997). Aminoglycoside kinases are peculiar in that they have a

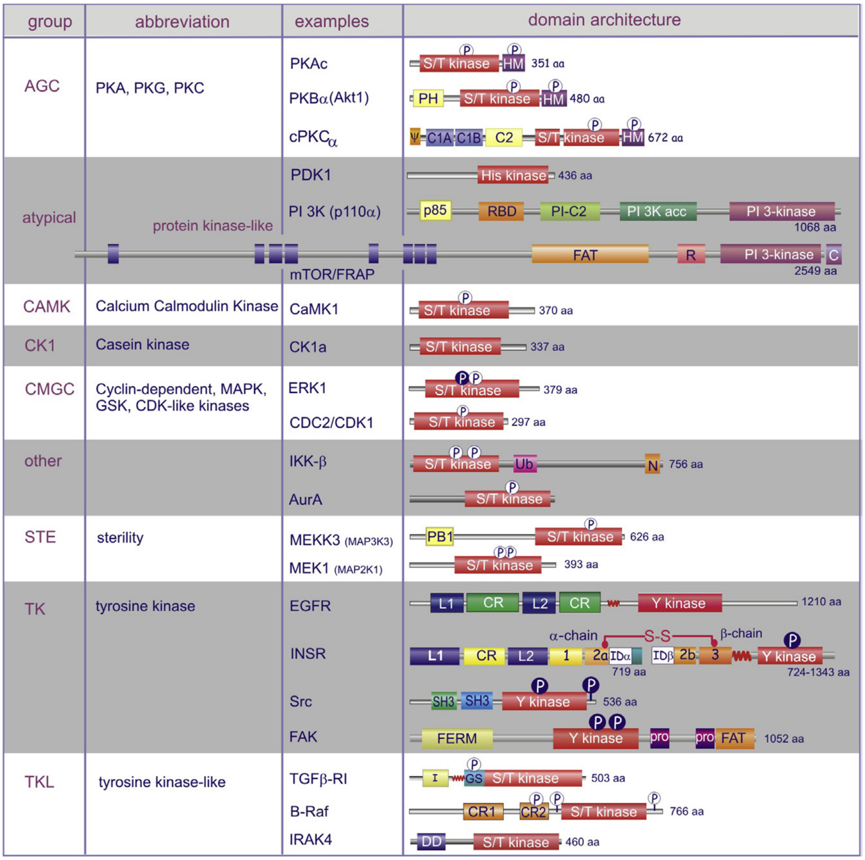


FIGURE 2-36 Protein kinase groups of the kinase superfamily and domain architecture of representative examples. Each group harbors one or more families of which some can be subdivided into subfamilies. Numerous abbreviations are explained in the text.

binding pocket for both ATP and GTP. For certain members of the family, the ATP pocket is not accessible and GTP is used for phosphoryl transfer (Smith et al., 2012). Aminoglycoside kinases (APH) are widely studied because they are responsible for resistance to the aminoglycoside antibiotic hygromycin B.

A superfamily of protein kinases

Protein kinases account for ~2% of most genomes and include over 500 separate genes in the human genome (Manning et al., 2002). The number is not exact because it is not straightforward to determine who belongs to the “superfamily” of protein kinases and who does not. The superfamily is divided in groups and these constitute one or more families. Protein families are characterized by proteins that shared a common ancestor gene, but have diverged over eons of evolution into distinct forms with similar but altered sequences. Sequence similarity normally suffices to constitute families. In some cases, however, the sequence divergence can occur to the point that simple sequence alignments no longer provide accurate information and other parameters must come into play such as structure and (if known) function. Most proteins have distinct 3D structures that determine much of their functional capability. Therefore, protein structure changes much more slowly than amino acid sequences. Structural similarity thus also provides powerful signals about the relationships between proteins. In other words, building accurate trees at the superfamily level requires both accurate sequence and structure details. In Figure 2-35, we show a sequence alignment of highly conserved motifs between two “typical” kinases, cPKA and GSK3, and one “atypical” kinase, PI3-kinase. All three catalyze phosphate-transfer and qualify as kinase but functional studies have revealed that PI3-kinase phosphorylates inositol-lipids rather than proteins. With respect to PI3-kinase, its belonging to the kinase superfamily would not immediately have become apparent by simply aligning its amino acid sequence with other typical kinases. Sequence alignment only shows 14 identical positions and 80 similar positions and, importantly, the comparison does not align the catalytic residues (aspartate). In fact, the catalytic residue of PI3-kinase is even not recognized. For comparison, when aligning the sequences of PKA and GSK3β, 80 identical positions are revealed, among which is the catalytic residue. Only when the sequence alignment is combined with conserved structural features does some similarity appear between atypical and typical protein kinases. The combined approach reveals the positioning of the catalytic aspartate (D*) residue in the β6 structure (at position 166 for cPKA). It also reveals some of the conserved amino acids involved in the coordination of ATP. They are either the same (as in β3) or similar such as in αC (glutamate replaced by aspartate), β4 (both leucine and isoleucine are used), and β5 (valine replaced by methionine). However, note that the α1, αF, and αI structures are lacking in PI3-kinase. These structures play a role in substrate recognition and this may explain why PI3-kinase phosphorylates inositol lipids rather than protein. It is postulated that atypical kinases are diverged early in evolution to form a distinct kinase group (Scheeff and Bourne, 2005). Atypical kinases are also devoid of a flexible loop near the catalytic cleft, also referred to as activation segment. The segment provides a framework for different regulation mechanisms comprising protein–protein interaction and phosphorylation. We will return to the activation segment when describing different activation mechanisms of protein kinases.

Based on four criteria (i.e., sequence similarity in the kinase domain, the presence of additional domains (noncatalytic), evolutionary conservation of the structure, and functional tests), the kinase superfamily is separated into 10 groups, each comprising one or more families (and sometimes subfamilies). The classification is not yet definitive for all families because new insights are still provided through new sequence and structural data. The groups are shown in Figure 2-36, ranked in alphabetical order (Cheek et al., 2002, 2005).

1. The AGC group is named after the protein kinase A, G, and C families (PKA, PKG, and PKC), which have a long history as cytoplasmic serine/threonine kinases that are regulated by secondary messengers such as cyclic AMP (PKA), cGMP (PKG), or Ca^{2+}/lipids (PKC). The group consists of 16 families of which 8 are likely to be of ancient origin as they are detected in all eukaryotes, with another two (RSK and PKC) in the fungal/metazoan lineage and six more (PKG, PKN, DMPK, YANK, RSKR, and RSKL) only found in metazoans. They share an activation mechanism where a C-terminal tail has to tether to a hydrophobic patch on the N-lobe and the activation segment has to be phosphorylated (see below for the regulation of activity of protein kinase A).
2. The atypical group contains the protein kinases of which the (predicted) structure does not resemble that of any other. Among these are the pyruvate dehydrogenase kinases (PDK) that are histidine kinases, pyruvate kinases (PyK), and nucleoside-diphosphate kinases (NDK). This group also contains the protein kinase-like (PKL) group of kinases that consists of several diverse kinase families that have the kinase-fold and catalytic mechanism, but do lack the refinements of the genuine ePK. Among these we find mTOR, ATM/ATR, as well as the phosphatidyl-inositol kinases such as PI3-kinase.
3. The CAMK group is a large group named after the Ca^{2+}/calmodulin-dependent protein kinases that operate in calcium-triggered signaling cascades and are involved in regulation of transcription, cell cycle progression, hormone production, cell differentiation, actin filament organization, and neurite outgrowth.
4. The casein kinase 1 (CK1) group comprises the casein kinases as well as the tau-tubulin brain-derived kinase (TTBK) and vaccinia-related kinases (VRK).
5. The CMGC group is named after the initials of some of its members. The group includes members of the cyclin-dependent kinases (CDK) that control the cell progression cycle, the MAPK growth- and stress-response kinases, Glycogen synthase and Casein kinase (glycogen metabolism), and protein kinases involved in hnRNA splicing (SRPK family). They are regulated by a unique mechanism that involves a phosphorylated tyrosine in the activation segment or a pre-phosphorylated tyrosine in the substrate. All substrates carry a proline adjacent to the target serine/threonine.

6. Members of the "other" group all contain a genuine eukaryotic protein kinase domain but the entire proteins do not fit into the major groups. Examples are the aurora (aur) family of mitotic protein kinases, potential targets for cancer therapy, IRE, localized in the rER and involved in the signaling of protein folding stress as well as I-κ kinase (IKK), a kinase that plays a key role in development (dorsal–ventral axis in *Drosophila*) and in the regulation of inflammation.
7. The sterility group (STE) is named after a set of genes in *Saccharomyces cerevisiae* (baker's yeast) required for mating between two haploid cell types (a- and α-cells). They act downstream of pheromones that arrest the mitotic cell cycle and induce mating-type-specific genes. They are part of the so-called MAPK growth- and stress-response kinases pathways.
8. The tyrosine protein kinases (TK) constitute an enormous group of kinases that phosphorylate (almost) exclusively on tyrosine residues, as opposed to most other kinases that are selective for serine or threonine. This group comprises two big families, the receptor tyrosine kinases (RTK) and the non-receptor (cytosolic) tyrosine kinases. They were discovered in search for oncogenes carried by viruses (Rous sarcoma virus or Ableson murine leukemia virus). We now know that these "oncogenes" are genes stolen from the host during an infection cycle, subsequently mutated by the virus and then reinserted in the host genome during another infection cycle. Examples are the cytosolic protein kinases sarcoma, Src, and focal adhesion kinase (FAK). Well known receptor-bound tyrosine-protein kinases are the epidermal growth factor receptor (EGFR) and the insulin receptor (INSR).
9. The tyrosine kinase-like group (TKL) is the most recently-defined and the most diverse of the typical kinase groups. Despite sequence characteristics of tyrosine kinases, they predominantly phosphorylate substrate on serine/threonine residues. Families within the group are relatively weakly related to each other but, as the name indicates, all are similar to members of the tyrosine kinase group. Over half of plant kinomes is made of TKL kinases and they include receptor kinases and possibly some genuine tyrosine-specific kinases. In animals, the receptors for the growth and transformation factor TGFβ, TGFβ-R, belong to this group.
10. Finally, the receptor guanylate cyclases (RGC). They form a tiny group of transmembrane proteins that contains an active guanylate cyclase domain, which generates the second-messenger cGMP. They belong to the protein kinase superfamily because they contain a catalytically inactive kinase-domain. They are omitted from Figure 2-36

Web resources about protein kinase families

- For more information about protein kinase groups: http://www.uniprot.org/docs/pkinfam.
- For more information about phosphoproteins in bacteria, yeast, *Caenorhabditis elegans*, *Drosophila*, mouse, and *Homo sapiens*, we refer to www.phosida.com.

TYROSINE KINASES IN UNICELLULAR ORGANISMS: BACTERIA AND PROTISTS

Tyrosine protein kinases have long been considered a hallmark of intercellular communication, unique to multicellular animals. The tyrosine kinase may be less abundant in bacteria but, over the last 10 years, biochemical, phosphoproteomic, and genomic analysis has revealed that bacteria contain phosphotyrosines and they contain at least three eukaryotic-like tyrosine kinases. A first clear example came from studies on *Acinobacter johnsonii*, which contains an 81 kDa protein, named Ptk, that autophosphorylates on tyrosine (Grangeasse et al., 1997). *Escherichia coli* also carries a protein tyrosine kinase, named Wzc (Vincent et al., 1999). An extensive genomic analysis of numerous strains has revealed that myxobacteria encode hundreds of eukaryotic-like kinases, among which are several tyrosine kinases. These bacteria have the particularity of carrying a relatively large genome, 13 mega base pairs, compared to some bacteria that carry as little as 0.2 mega base pairs. Moreover, they are able to aggregate with the purpose of concentrating extracellular enzymes that are used to digest food. The process of regulating population density, through the production of chemotactic stimuli, is referred to as quorum-sensing (Pérez et al., 2008). Phosphoanalysis showed the presence of phosphotyrosine in *E. coli*, *Bacillus subtilis*, and *Lactococcus lactis* (reviewed in (Macek and Mijakovic, 2011)). Protists, unicellular eukaryotes that are considered to be the closest known relatives of multicellular animals (metazoans), also express tyrosine kinases. One of its members, the choanoflagellate *Monosiga brevicollis*, has a remarkable count of 128 and it possesses a tyrosine-kinase signaling network more elaborate than found in any known metazoan (Manning et al., 2008; Suga et al., 2012). It is suggested that receptor tyrosine kinases that had been used for receiving environmental cues in unicellular organisms were subsequently used as a communication tool between cells at the onset of metazoan multicellularity.

PROTEIN DOMAINS, THEIR FOLDS, AND THEIR GRAPHIC REPRESENTATIONS

The amino acid chains of structured proteins fold into one or several domains. Each domain forms a compact 3D structure and often can be folded independently and be stable by itself. Often, protein domains can be equated with a particular function. Protein-interaction domains, of which 85 have been recognized today, play an important role in assembly of cell signaling networks by binding to other protein-domains, to lipids or nucleic acids. In a linear presentation, domain architecture is often displayed in the form of cylinders or boxes and they are indicated by abbreviations (SH3 for Src-homology regions or CR1 for cysteine-rich region-1). Highly conserved nonstructured regions, called motifs, are also depicted in typical domain architecture presentations as shown in Figure 2-37. The catalytic domain of human protein kinase A (PKAc, gene name PRKAC) for instance has one domain, the kinase domain (255 amino acids). It is preceded by a poorly structured sequence of 44 amino acids and followed by a conserved hydrophobic motif (HM) (also known as AGC-kinase motif) of 53 amino acids. In contrast, protein kinase C (gene name PRKC) carries multiple domains connected by (unstructured) linker regions. Starting at the N-terminal we first find the psi motif, a pseudosubstrate that plays a role in regulation of the catalytic domain, this is followed by two conserved (cystein-rich) sequences, the C1A and C1B domains (each 51 amino acids), which bind diacylglycerol in the cell membrane. The sequence continues with another conserved (cystein-rich) C2 domain (89 amino acids), which plays a role in Ca^{2+}-mediated phospholipid binding, and a catalytic domain (259 amino acids). The sequence ends with a conserved HM of 71 amino acids. The flexible linkers make this a rather flexible protein. Figure 2-37 shows how the amino-acid sequence relates to the linear domain architecture of protein kinase C and how the two relate to the 3D structure of the protein. We depict an active protein kinase C, where C1A, -B and C2 are buried in the membrane and the pseudosubstrate motif is removed from the catalytic cleft. Like most members of the AGC kinases, the active protein kinase is phosphorylated in the HM region and in its activation segment (indicated by the P symbol in the domain architecture).

Phylogenetic studies have shown that increased complexity of organisms, meaning organisms with an increased number of different anatomical structures (organs, tissues), corresponds to an increased complexity of domain architecture of proteins. Proteins composed of multiple domains augment the number of regulatory sites and interactions with other proteins; in other words, they make protein networks more diverse and more integrated (Anantharaman et al., 2007).

INFORMATION ABOUT PROTEIN (INTERACTION) DOMAINS ON THE WEB

- Prosite for all protein domains http://prosite.expasy.org/.
- Pawson lab for protein interaction domains- http://pawsonlab.mshri.on.ca.
- Cell signaling technology for protein interaction domains http://www.cellsignal.com/reference/domain/index.html.

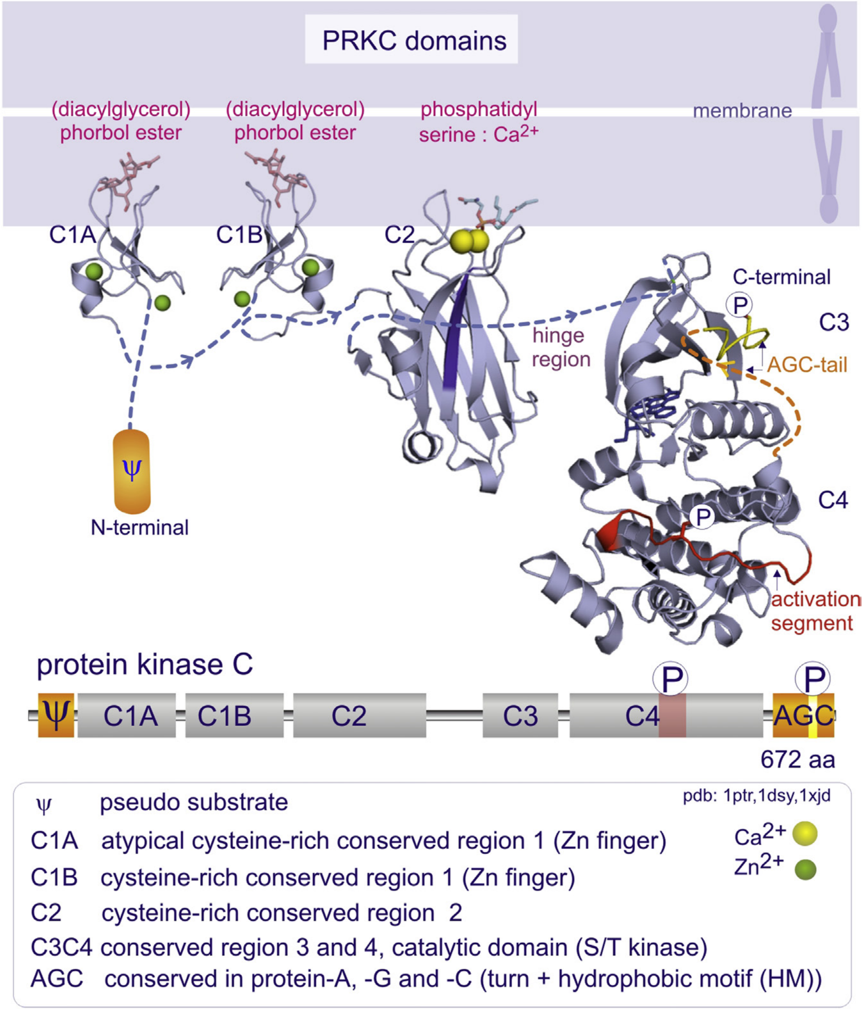

PRKC domains
(diacylglycerol) phorbol ester
(diacylglycerol) phorbol ester
phosphatidyl serine : Ca2+
membrane
C1A
C1B
C2
C-terminal
P
C3
hinge region
AGC-tail
ψ
N-terminal
C4
P
activation segment
protein kinase C
P
P
ψ
C1A
C1B
C2
C3
C4
AGC
672 aa
ψ pseudo substrate
pdb: 1ptr,1dsy,1xjd
C1A atypical cysteine-rich conserved region 1 (Zn finger)
Ca2+ ●
Zn2+ ●
C1B cysteine-rich conserved region 1 (Zn finger)
C2 cysteine-rich conserved region 2
C3C4 conserved region 3 and 4, catalytic domain (S/T kinase)
AGC conserved in protein-A, -G and -C (turn + hydrophobic motif (HM))

FIGURE 2-37 Representation of protein domains in a 3D structure and in a linear domain architecture diagram. The example is human protein kinase Cα a multidomain protein, here represented in its active state. Its C1A, -B and C2 domains are buried in a lipid bilayer (due to the presence of phorbol ester and Ca^{2+}), the pseudo-substrate ψ is removed from the catalytic domain and the catalytic- and HM-domains are phosphorylated. The structures shown are a compilation of conventional and novel PKC isoforms: C1(A+B) of PKCδ, C2 of PKCα, and C3/C4 from PKCθ. pdb: 1ptr, 1dsy, 1xjd.

WHICH AMINO ACIDS ARE SUSCEPTIBLE TO PHOSPHORYLATION?

Serine, threonine, and tyrosine

There are nine amino acids that can be phosphorylated but only three of them give stable, phospho-ester, bonds and these are serine, threonine, and tyrosine. With respect to their relative abundance, analysis of cells kept in culture has revealed that phospho-serine makes up 86%, phosphothreonine 12%, and phosphotyrosine 2% (Olsen et al., 2006; Hunter and Sefton, 1980). Until today, these amino acids are the key targets in all known signaling events. In the majority of cases, tyrosine kinases do not phosphorylate serine or threonine residues and vice versa. Selectivity is not yet fully understood but there are probably several underlying mechanisms. One rather straightforward explanation comes from the observation that serine and threonine are simply too short in order to bring their hydroxyl group near the catalytic aspartate and γ-phosphate of a "tyrosine protein kinase." However, dual-specificity protein kinases do exist, they phosphorylate on serine, threonine, and tyrosine. An example is the family of dual-specificity mitogen-activated protein-kinase kinase (MAP2K or MEK) that act downstream of growth factor receptors. They belong to the STE group, see the example of MEK1 in Figure 2-36.

Aspartate and glutamate

The carboxylate anion of glutamate and aspartate can also be phosphorylated but the anhydride-bond is unstable under all conditions and therefore difficult to study. Phospho-glutamate and phospho-aspartate have a substantially greater thermodynamic propensity to transfer their phosphate onto water (or another nucleophile) than does ATP. Aspartate and glutamate kinases have not yet been purified but phospho-aspartate deserves further attention because it drives the structural changes and the rhythmicity of the Ca^{2+}-pump (SERCA) in the sarcoplasmic reticulum. When two calcium ions are bound inside the transmembrane domain of the pump, ATP attaches to the P-domain and this leads to a minor shift in the cytoplasmic P- and A-domains. Following ATP binding, aspartate-351 immediately gets phosphorylated. Upon the subsequent loss of ADP, the A subunit makes a 90° turn that causes the "ejection" of the calcium ions into the lumen of the sarcoplasmic reticulum. The subsequent hydrolysis of the labile anhydride bond resets the pump to its start position. The cycle turns fast when intracellular free Ca^{2+} is elevated and slows down when resting levels are reached (around 100 nM). SERCA does not qualify as a eukaryotic protein kinase (Toyoshima, 2009).

Cysteine

The thiol group (-SH) of cysteine can be phosphorylated, thus forming a thioester. There are no examples for eukaryotes but cysteine is phosphorylated in bacteria (see below in the section about phosphoenolpyruvate and glucose transport)

Histidine, arginine, and lysine

Histidine, arginine, and lysine can be phosphorylated on their amino group, in the case of histidine there are two sites (N1 and N3), but the phosphoramidate bond is labile (phosphohistidine have a large standard free energy of hydrolysis) (Figure 2-38). To our knowledge arginine and lysine kinases have not yet been identified in eukaryotes. With respect to histidine kinases, they seem to be largely reserved for prokaryotes but eukaryotes do have two small families: the PDK (counting four members) and the methyl-oxobutanoate dehydrogenase kinase (BCKDK, counting only one member). With respect to human PDKs, whereas their amino acid sequences resemble prokaryotic histidine kinases, PDK3 phosphorylates and activates pyruvate dehydrogenase on serine residues. On the contrary, nucleoside-diphosphate kinase-A and -B (NDK-A and -B) are kinases that autophosphorylate on histidine but, unfortunately, their sequences resemble more those of tyrosine protein kinases. Human nucleoside-diphosphate kinases (NDK) catalyze the transfer of the γ-phosphate from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP) by using ATP as a major phosphate donor. During the catalytic reaction, the enzymes are transiently phosphorylated (creating an enzyme intermediate state) on a conserved histidine residue (H118) (Devedjiev et al., 2007). Finally, we mention that histine protein kinases share structural homology with the ATP-binding regions of the heat-shock protein Hsp90 and DNA gyrase B (PDB 2xcs).

BACTERIAL EXCEPTIONS: PHOSPHOENOLPYRUVATE AS PHOSPHATE DONOR AND HISTIDINE KINASES AS ENVIRONMENTAL SENSORS

PEP features in the list of Table 2-5 as a possible source for the transfer of phosphate. Bacteria use it in an ingenious way in nutrient signaling pathways. We explore one example, the regulation of glucose metabolism in *E. coli*. Regulation occurs by a protein complex comprising a phosphotransferase (EI) and three substrates, namely HPr, EIIA, and the cytosolic domain (IIB) of the membrane glucose-transporter IIBC (Figure 2-39). The ensemble of proteins is referred to as the PEP-dependent

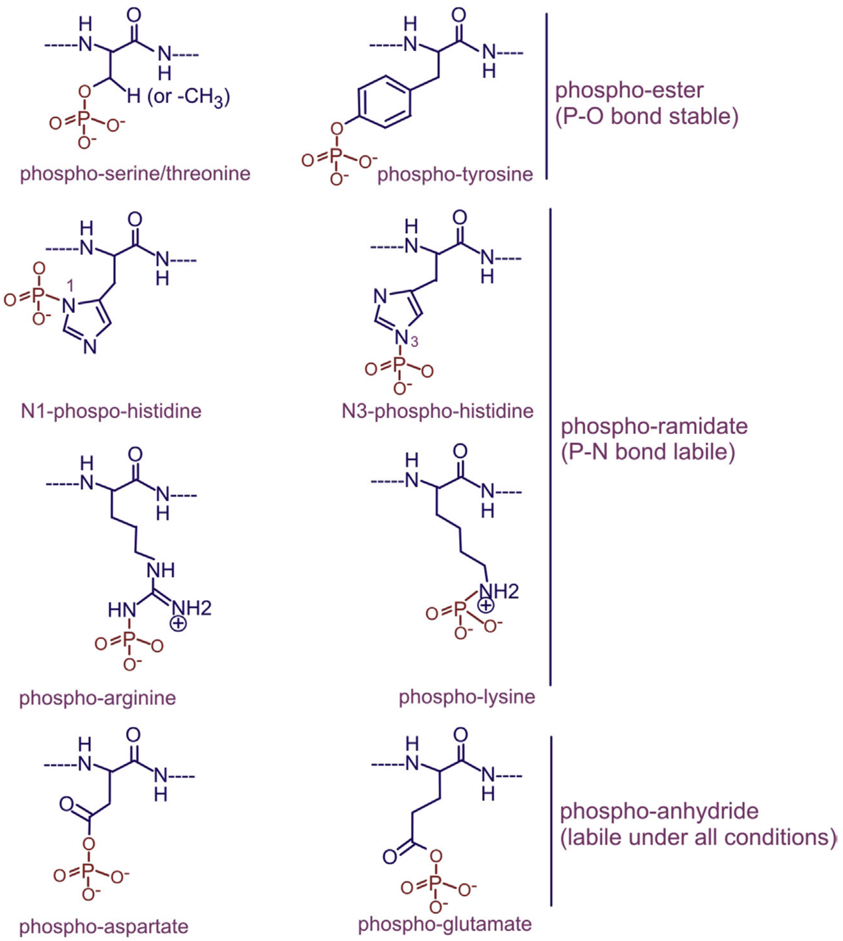


FIGURE 2-38 Different phospho-amino acids. Only the phospho-ester bonds of phosphoserine, -threonine, and -tyrosine form stable bonds. Phospho-ramidate and -anhydride are labile, occurring for very short times and therefore hard to study.

phosphotransferase system (PTS) and it represents an interesting data-processing network that combines food uptake with gene expression. The PEP transferase EI transfers the phosphate on itself, more precisely on its histidine-189 residue. Despite the fact that it transfers phosphate on a histidine, it does not qualify as a histidine protein kinase. From here a phosphorelay ensues in which the phosphate first transfers onto histidine-15 of HPr, then jumps onto histidine-90 of EIIA, and finally settles on cysteine-35 of the cytosolic domain (EIIB) of the transporter. When glucose crosses the transporter, phosphate transfers onto the sugar so as to create glucose-6-phosphate. Like in eukaryotes, glucose-6-phosphate

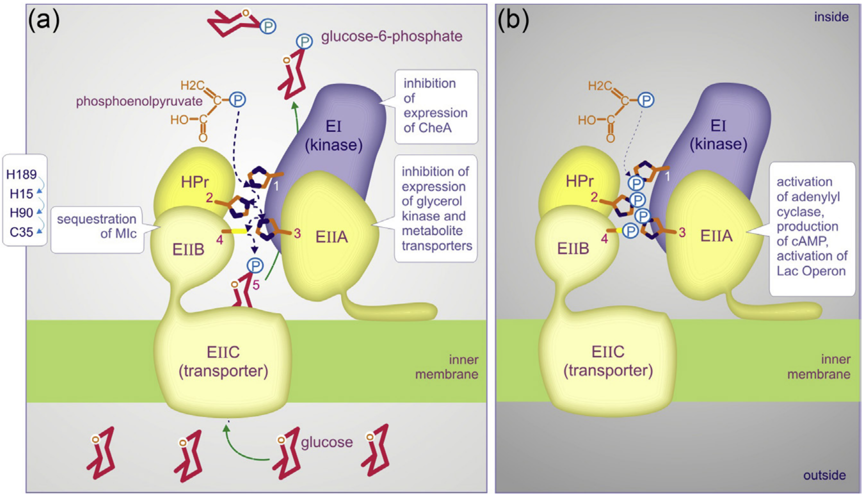

(a)
glucose-6-phosphate
H2C
phosphoenolpyruvate
HO
inhibition of expression of CheA
EI (kinase)
inhibition of expression of glycerol kinase and metabolite transporters
H189
H15
H90
C35
sequestration of Mlc
HPr
EIIB
EIIA
1
2
3
4
5
EIIC (transporter)
inner membrane
glucose
(b)
inside
activation of adenylyl cyclase, production of cAMP, activation of Lac Operon
outside

FIGURE 2-39 Regulation of glucose metabolism by the phosphoenolpyruvate-dependent phosphotransferase system. (a) Glucose enters the bacterium through the membrane transporter EIIC. The phosphorylation of glucose acts as a sink for the phosphate relay therefore keeping the transfer system underphosphorylated. The underphosphorylated components suppress expression of CheA (a protein that stimulates rotation of the flagellar motor) and of metabolite transporters other than for glucose. MIc, a suppressor of glucose metabolism components, is kept away from the DNA through sequestration by EIIB, (b) The situation is reversed in the absence of glucose, all the proteins are phosphorylated. As a consequence the glucose pathway is switched off, the bacteria “start” their flagellar motor proteins and the Lac Operon is activated by EIIA (allowing a switch to lactose metabolism).

enters the glycolytic cycle (glycolysis), which leads to the production of PEP. When glucose is abundant, the above-mentioned proteins will be predominantly in their nonphosphorylated state (Figure 2-39(a)). The dephosphorylated EI inhibits expression of CheA, a protein that normally stimulates rotation of the flagellar motor. Dephosphorylated EIIA inhibits expression of glycerol kinase as well as a variety of other metabolite transporters. The dephosphorylated EIIB sequesters Mlc, a repressor of genes that code for glucose transporters as well as enzymes involved in glucose metabolism. In short, the bacteria focus on glucose transport and metabolism and shut down alternative metabolic pathways. When the entry of glucose falters and phosphate is no longer removed effectively from EIIB, the proteins accumulate in their phosphorylated state. As a consequence chemotaxis commences (renewed expression of CheA) and glucose transporters and glucose-metabolizing enzymes are down-regulated. Moreover, the phospho-EIIA protein activates adenylate cyclase, which through the production of cAMP leads to activation of the Lac operon and the bacteria switch to lactose metabolism (when they are in the gut) (Figure 2-39(b)) (Cai et al., 2003).

PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEM

A pioneer study of phosphoenolpyruvate phosphotransfer (Kundig et al., 1964).

Bacteria have developed numerous sensors that probe the environment and orchestrate appropriate responses for survival. A number of sensors are receptor histidine-protein kinases (HK), enzymes that function as homodimers. Histidine kinases are a separate class of kinases because they have a unique kinase-fold. They are recognized by short conserved sequences in the catalytic/ATP-binding domain. The dimeric sensors receive and transmit extracellular signals into the cell through phosphorylation of one histidine residue each (this occurs either through *trans*- (neighbor) or *cis*-(auto) phosphorylation) (Figure 2-40). The sensor is linked to response regulators (RR), the equivalent of "transducers" in eukaryotic cells. Receptor and RR constitute a "two-component system." The RR are modified through a phosphotransfer onto a highly conserved aspartate. This alters their conformation and causes dimerization with a concomitant change in binding efficacy to promoter regions of genes. Receptors and regulators comprise a "two-component system" (TCS). It is estimated that as many as 100 different TCSs exist in a single bacterium (Casino et al., 2010; Jung et al., 2012). While histidine kinases have also been identified in archaea, fungi, ameba, and plants, the number of homologous genes in eukaryotic genomes is much lower than those found in bacterial genomes (Kim and Forst, 2001).

As a specific example of a two component system we elaborate on EnvZ, an osmolarity sensor. It is expressed in *E. coli* (strain K12) and forms a dimer that is inserted in the inner membrane. Increased osmolarity (of the environment) alters the relative position of the His-kinase domain and the histidine-243 autophosphorylation site, allowing histidine phosphorylation (either in *cis* or in *trans*). The phosphate is next transferred to aspartate-55 of OmpR, a protein that shuttles between DNA and the EnvZ dimer. OmpR is a transcription factor that controls expression of the outer membrane proteins C and F (OmpC and -F). These proteins allow the passive diffusion of nutrients, water, and ions. OmpF is more conducive (bigger pore) and used preferably when the environment is low in nutrients (and thus low in osmolarity). Once osmolarity rises, OmpC expression dominates over OmpF. OmpR binds both promoters but in its dephosphorylated state it is more effective for OmpF. Phosphorylation of OmpR modifies dimer formation and this increases binding to the OmpC promoter whereas it somehow blocks expression of OmpF (Yoshida et al., 2006; Barbieri et al., 2013). Finally, it should be noted that at low osmolarity, the histidine kinase exhibits phosphatase activity.

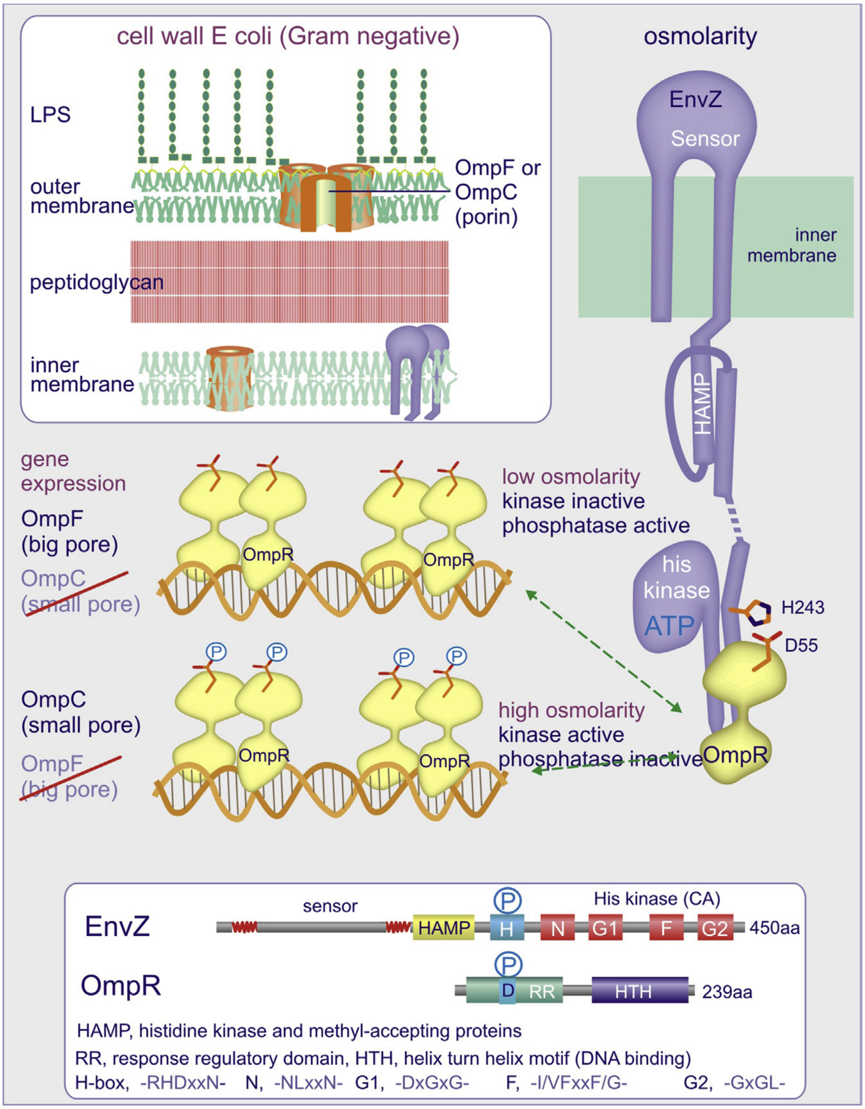


FIGURE 2-40 Receptor histidine kinase and two-component signaling system in bacteria.

SUBSTRATE PHOSPHORYLATION MOTIFS AND DISTAL DOCKING SITES

Linear phosphorylation motifs

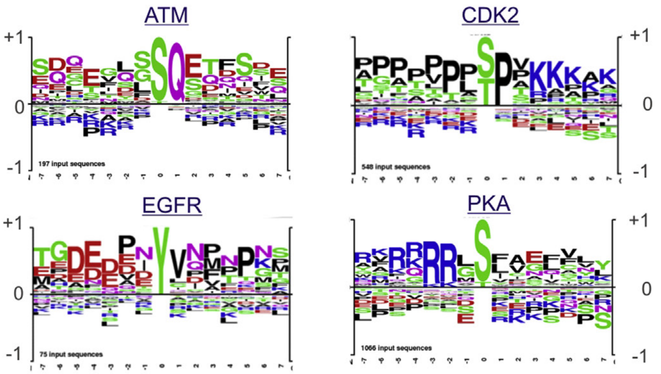
Substrate recognition by protein kinases is mediated by the recognition of the primary amino acid sequence (linear motif) surrounding the phosphorylation site (phosphosite) and by two contextual elements: the presence of distal substrate docking sites on the protein kinase and the presence of scaffold proteins that determine subcellular localization and/or substrate proximity.

Phosphosites have been studied in two ways; by presenting protein kinases with a large range of short peptides, and study their degree of phosphorylation (peptide-library screening) and by analysis of the composition of phosphosites of partially digested proteins by mass spectrometry. The phosphoproteins are either obtained in vivo, from cells treated with a hormone or a growth factor, or obtained in vitro, after having mixed a purified kinase with a mix of substrates (Mok et al., 2010; Amanchy et al., 2007; Monetti et al., 2011). A phosphosite is described in terms of which amino acids are frequently present at which positions relative to the site of phosphorylation. As an example, the sequence motif for protein kinase A has been determined as -R-R-x-S/T-x- (where x represents any amino acid) (Kemp et al., 1977). At a more complex level, the specificity can be described using a position-specific scoring matrix wherein the probability of each amino acid at each position relative to the site of phosphorylation is given (Figure 2-41). These descriptions are practical and readable, but they are generally neither sufficient nor necessary descriptions of a kinase's substrates. The current line of thinking is that, with some exceptions, substrate specificity is rather degenerate (both for protein kinases and phosphatases). Protein kinases seem to have a preference for disordered regions which allow the kinases to mold them to an extended conformation that fits the catalytic site. As a consequence, substrate specificity must come largely from inclusion of contextual elements (see below) (Alexander et al., 2011; Amanchy et al., 2007; Obenauer et al., 2003). It is important to realize that protein kinases do not always have an unlimited choice; they may not necessarily meet their favorite substrate or, the other way round, nonfavorable substrates may impose themselves because they are part of the same multiprotein complex. It is worth noting that because of the degeneracy of phosphorylation motifs, numerous phosphorylation sites could occur on proteins that have no or little functional relevance. Identification of functional phosphorylation networks should therefore draw upon evolutionary information. Indeed, phosphorylation sites of known function are more conserved than phosphorylation sites of unknown function (Landry et al., 2009).

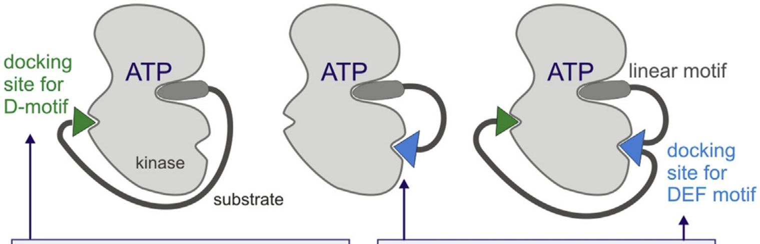
Web resources for analysis of linear phosphorylation motifs

http://www.phosphosite.org/homeAction.do.
http://scansite3.mit.edu/#home.
http://phospho.elm.eu.org/ for S/T/Y phosphorylation sites.
http://netphorest.info/ for more information about netphorest (Miller et al., 2008).

(a) linear motifs (phosphosites)



(b) docking sites on ERK (mitogen activated protein kinase)



proteins with a docking motif (D-motif)	
upstream kinase	
MEK1	-KKKPTPIQLNP-
MEK2	-RRKPVLPALTI-
downstream kinase	
RSK1	-LAQRRVRKLPS-
MNK1	-LARRRALAQA-
regulatory phosphatase	
MKP3	-RRLQKGNLPVR-
MKP4	-RRLRRGSLSVR-
MKP7	-RRLQQDKVLIT-
transcription factor	
ELK-1	-RKPRDLELPLSP-

proteins with a FxFP sequence (DEF motif)	
scaffold protein	
KSR-1	-FSFP-
transcription factor	
c-Fos	-FLFP-
ELK-1	-FQFP-

(c) sequence of human Elk-1 with docking sites and phosphosites

1 **MDPSVTLWQF LLQLLREQGN GHIISWTSRD GGEFKLVDAE EVARMWGLRK NKTNMNYDKL**
60 **SRALRYYYDK NIIRKVSGQK FVYKFVSYPE VAGC**STEDCP PQPEVSVTST LPNVAPAAIH
120 AAPGDTVSGK PGTPKGAGMA GPGGLARSSR NEYMRSGLYS TFTIQSLQPQ PPPHPRPAVV
180 LPSAAPAGAA APPSGSRSTS PSPLEACLEA EEAGLPLQVI LTPPEAPNLK SEELNVEPGL
240 GRALPPEVKV EGPKEELEVA GERGFVPETT KAEPEVPPQE GVPARLPAVV MDTAGQAGGH
300 AASSPEISQP QKG**RKPRDLE LPLSP**SLLGG PGPER**T**PGSG SGSGLQAPGP AL**T**PSLLPTH
360 TL**T**PVLL**T**PS SLPPSIHFWS TL**S**PIAPR**S**P AKLS**FQFP**SS GSAQVHIPSI SVDGLS**T**PVV
420 L**S**PGPQKP

DNA binding domain, D-motif, DEF-motif
phosphosites reported by PhosphoSite (sites supported by 5 references)
additional phosphosites reported by SwissProt (accession P19419)

FIGURE 2-41 Linear phosphorylation motifs and distal docking sites. (a) Protein kinases recognize substrate by linear phosphorylation motifs (phosphosites). Phosphorylation sequences have been analyzed for large numbers of substrates and these are presented as a Position-Specific Scoring Matrix also known as the "sequence logo." Positive scores indicate that the given amino acid occurs more frequently in the alignment than expected

by chance, while negative scores indicate that the substitution occurs less frequently than expected. Large positive scores indicate critical residues required for substrate recognition by the protein kinase in question. Note that the sequences surrounding the phosphorylation sites are not very stringent. The protein kinase ATM requires substrates with a serine followed by glutamine (–S–Q–) whereas CDK2 requires a serine or threonine followed by proline (–S/T–P–). For PKA the concensus would be two arginines followed by a nonpolar and a serine (R–R–x–S–) but no clear preferences are discernable for the EGF receptor. Data obtained from www.phosphosite.org (b) Distal docking sites also play a role in the choice of substrates. The serine/threonine protein kinase ERK2 has two docking sites, one is positioned at the "back" of the protein kinase and recognized by proteins carrying a D-motif, and the other positioned just underneath the catalytic cleft, and recognized by proteins with a DEF motif. It is not clear yet in whether or not binding occurs in an ordered manner that is, first docking site interaction and then binding of the linear motif in the catalytic cleft, (c) The sequence of the transcription factor ELK-1 contains both a D-motif (in green) and a DEF-motif (in blue). Thus it can attach to ERK at two different sites. The DNA-binding sequence is shown in orange. The protein features numerous residues phosphorylated by ERK kinases. The ones reported by PhosphoSite, with at least five references, are shown in purple. Additional sites reported in UniKprot (accession number P19419) are shown in dark blue. Other phosphorylation sites exist (but not yet confirmed by independent studies).

Distal docking sites for substrates

Distal docking sites play an important role in the interaction between protein kinases and their substrates. They occur on the catalytic domain itself, on regulatory subunits or they occur on additional protein-interaction domains carried by the protein kinase. Docking determines subcellular localization, binding to a scaffold protein at a specific location within the cells, but they also play an important role in selecting substrates and in rendering the phosphorylation process more efficient. Distal docking sites determine the velocity and the amplitude of the response (Welch et al., 2010; Reményi et al., 2006).

A well-studied example is ERK2, a key protein kinase involved in growth factor signaling (mitogen-activated protein kinase (MAPK)). It contains two docking sites, one at the back of the catalytic domain, and named common docking site (CD), and one just underneath the catalytic cleft. The common docking site is recognized by an arginine or lysine and at least two nonpolar or uncharged amino acids (Figure 2-41). It binds proteins that regulate the activity of ERK2 (upstream in the signaling cascade) and proteins that are regulated by ERK2 (downstream substrates). One of the regulators is the phosphatase MKP3; it dephosphorylates and inactivates ERK2. One of the substrates is the transcription factor ELK-1; it gets phosphorylated in the nucleus. The second binding site, underneath the catalytic cleft, is recognized by a cytosolic scaffold protein, KSR-1, and by two transcription factors, c-fos and (again) EKL-1. KSR-1 has three subcellular locations: it is found in the cytoplasm, sequestered by the 14-3-3 protein, at the plasma membrane or at the endoplasmic reticulum. Proteins that recognize this docking site carry a short sequence containing two phenylalanines and one proline (motif FxFP). Understand well that proteins that recognize the same docking site are in competition with each other. This means that their relative expression levels as well as the subcellular localization of ERK2 (in the cytoplasm or nucleus) may affect the composition of the ERK2-protein complexes and thus the outcome of the phosphorylation cascade; the favorite substrate is thus determined by both a favorable (linear) phosphorylation motif and by a favorable docking situation.

The importance of docking was shown in the late 1970s with the discovery that both prostaglandin E1 and isoprotenolol (adrenalin agonist) cause an increase in cAMP in the heart, yet only isoprotenolol increased glycogen phosphorylase activity and the rate and force of contraction (Hayes et al., 1979). Somehow local hotspots of cAMP and PKA must combine the different receptors with different effectors. Two docking mechanisms have been discerned for the adrenaline to glycogen phosphorylase pathway. The first concerns docking of PKA and the second of phosphorylase kinase (Figure 2-42). With respect to PKA, the situation is unique in that the docking does not involve the catalytic subunit (cPKA) but the regulatory subunits (RI or RII). They dock onto scaffold proteins of the A-kinase-associated proteins (AKAPs). In the example of the skeletal muscle, AKAP250 (also known as AKAP5) situates the kinase/regulator complex next to adenylyl cyclase and the β2-adrenergic receptor (β2AR) whereas muscle -AKAP (mAKAP or AKAP6) situates the kinase complex next to the RyR Ca^{2+} channel. AKAP250 facilitates desensitization of the β2AR because it facilitates PKA-mediated phosphorylation and subsequent binding of arrestin (and this blocks interaction between G-proteins and their effectors) (Daaka et al., 1997; Zhang et al., 2011). Muscle -AKAP (AKAP6) enhances PKA-mediated phosphorylation of the RyR which augments the flow of Ca^{2+} and hence increases muscle contraction. However, mAKAP also binds phosphodiesterase-4, an enzyme that converts cAMP into AMP and, in doing so, terminates the adrenaline receptor signaling pathway. Certain isoforms of PDE4 are phosphorylated and activated through PKA (Dodge et al., 2001; Fischmeister et al., 2006). With respect to phosphorylase kinase, in the liver it is found associated with a large scaffold protein called GL. This protein also binds glycogen particles and associates with glycogen phosphorylase. Substrate, enzyme, and the upstream regulating protein kinase are grouped together and this renders regulation fast and effective. Muscle has two glycogen-associated proteins, Gm and a GL-like but how they combine different enzymes is not yet fully elucidated (Munro et al., 2002). With these two examples we wish to transmit the message that positive and negative regulators of signaling and metabolic pathways often cluster around scaffold proteins and together they form sophisticated centers of signal integration. We are not yet in the position to provide a clear model of how these feedback loops function exactly because too many parameters are not yet properly sought out.

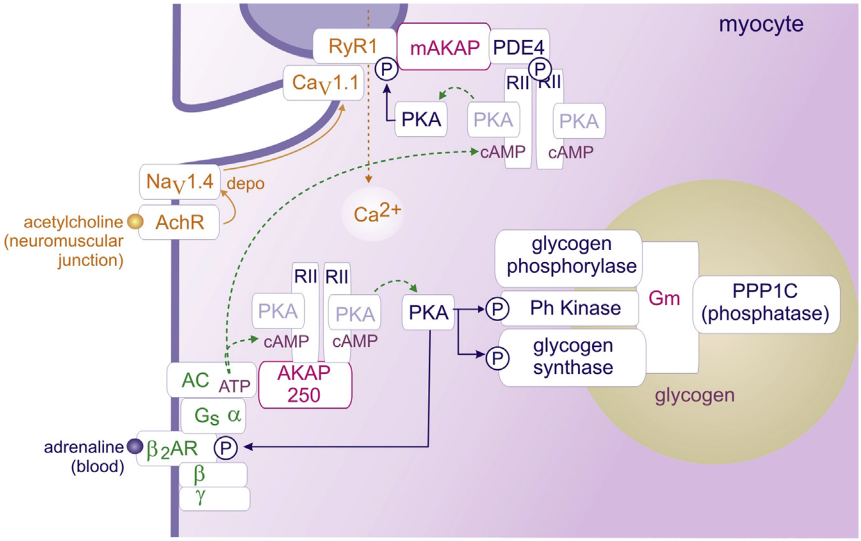


FIGURE 2-42 Docking motifs in the adrenaline to glycogen-phosphorylase pathway. The catalytic subunit of protein kinase A (PKA) is kept inactive by a regulatory subunit (RII). The production of cAMP by adenylyl cyclase (AC) liberates and activates the kinase. Regulatory subunits are bound to A-kinase-associated proteins (AKAPs) and these serve to localize the protein kinase at specific sites. AKAP250 positions the regulatory subunit/kinase complex near the β2 adrenergic receptor whereas muscle -AKAP warrants localization adjacent to the RyR1 Ca^{2+}-channel. The enzymes that regulate glycogen metabolism, glycogen phosphorylase, and glycogen synthase, are also attached to a scaffold protein, Gm, together with phosphorylase kinase, the enzyme that activates glycogen phosphorylase. Gm also binds glycogen. AKAPs and Gm control feed-forward as well as feedback processes. In the example shown, mAKAP facilitates phosphorylation of RyR1, causing an increase in Ca^{2+} flow which increases muscle contraction and glucose-1-phosphate production, and Gm facilitates phosphorylase kinase-mediated phosphorylation and activation of glycogen phosphorylase (also leading to more glucose-1-phosphate). In contrast, AKAP250 helps to phosphorylate and desensitize the β2AR and, by binding PDE4, mAKAP stimulates conversion of cAMP into AMP. These processes reduce the concentration of second messenger and attenuate the cellular response.

PROTEIN KINASE ACTIVATION MECHANISMS

In the section of posttranslational modifications we have already hinted to the fact that protein kinases are controlled. The example we gave dealt with the control of activity of the tyrosine protein c-Abl by its own C-terminal tail to which a myristate is attached (return to Figure 2-21(b)). The protein kinase-fold is pliable and a large majority of protein kinases requires an activation process in order to achieve catalytic competence (they are by default in an inactive state). This either occurs through phosphorylation or through interaction with other proteins or through interactions with specific domains within the protein kinase. Remember that protein kinases may display substantial structural difference in their non-active confirmation, in their competent (or active) state they resemble each other a lot. Returning to the structure shown in Figure 2-35, whatever the modifications, the activation process comprises the precise positioning of the crucial amino acids involved in coordination of the β- and γ-phosphate of ATP and of the target amino acid. The substrate must be oriented so that the hydroxyl is directed toward the catalytic aspartate (D166) while also being in contact with the γ-phosphate. Moreover, for serine/threonine kinases, the γ-phosphate must also contact a lysine residue, two amino acids away from the catalytic residue. The positively charged lysine is poised to stabilize the developing local negative charge during catalysis. In tyrosine kinases, the γ-phosphate makes contacts with an arginine, which positions four amino acids away from the catalytic aspartate, thus allowing space for the much larger tyrosine substrate residue. In addition, a set of conserved hydrophobic residues, including the adenine moiety of ATP, must be positioned such that they create two spines that stabilize the relative positioning of the C- and N-lobe (Ten Eyk et al., 2008). Once these conditions are met, the kinase will execute phosphotranfers at the rate of tens per second. A particular flexible segment of the kinase domain, referred to as the "activation segment" or the "activation loop," takes central stage in the activation process. It is strategically positioned in the catalytic cleft and affects both substrate binding and the organization of the N- and C-lobe and as a consequence the coordination of ATP (Figure 2-35). It often must undergo substantial displacement in order to render protein-kinases catalytically competent, hence its name. Apart from the above-described core elements of the catalytic domain, both C- and N-tails of the kinase domain contribute to the regulation of activity. These can be short loosely structured sequences that wrap around the catalytic domain (case of PKA and CDK2) or highly structured domains (case of c-Abl, PKC, PKG). These sequences also play a role in subcellular localization (two essential reviews: Taylor et al. (2012b), Endicott et al. (2012)).

Below we will give a number of examples of how protein kinases are rendered competent (how they are activated). Just after synthesis, when the PKA is still bound to the ribosome, it phosphorylates itself at a serine-338 residue near the C-terminus, in the so-called AGC motif, characteristic of AGC protein kinases. This phosphorylation packs the C-terminus against the N-lobe and affects the position of the αC-helix. A second phosphorylation occurs at threonine-197, in the activation segment, this time by another protein kinase, either another PKA or by a kinase named PDK1 (phospholipid-dependent protein kinase-1). The two phosphorylations are somehow insensitive to protein phosphatases and they seem not to be a target for inactivation mechanisms. The phosphothreonine-197 not only pushes the activation segment outward, and leaves room for the binding of substrate, but also sets in train the precise positioning of two conserved amino acids (Figure 2-43) involved in the coordination of Mg^{2+}/ATP. One concerns the orientation of the conserved glutamate in the αC-helix of the N-lobe (E91) and the other a shift in the orientation of aspartate (D184) in the conserved -DF- motif in the β8-helix of the C-lobe (this change in position is referred to as the "DFG flip"). Once competent, the catalytic domain is bound by a regulatory subunit (RIα in Figure 2-43), which blocks the kinase by acting as substrate. Depending on the type of regulatory

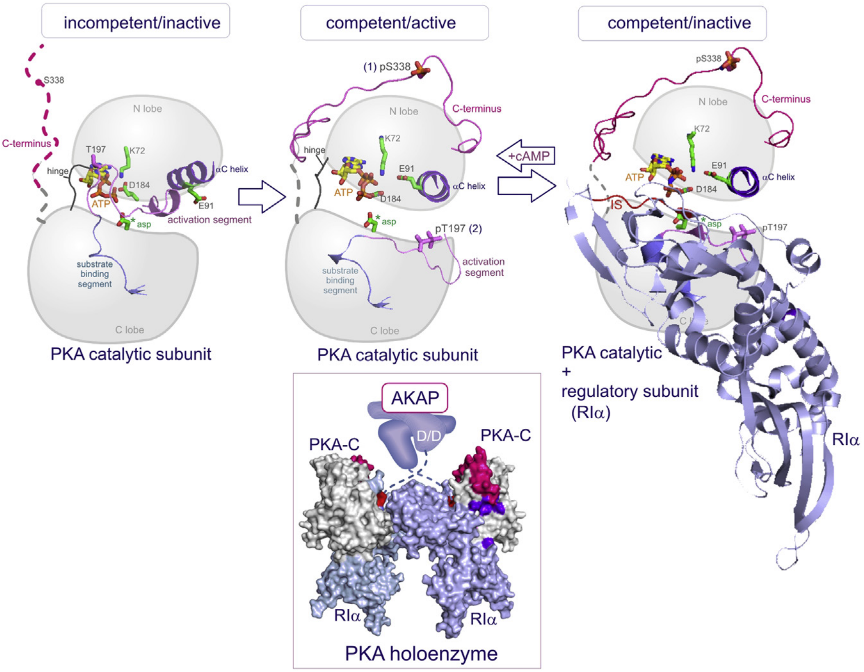


FIGURE 2-43 Regulation of protein kinase A in three stages. The protein kinase is synthesized in a catalytically incompetent conformation. Two phosphorylation steps follow immediately upon synthesis, one in the C-terminus (Ser338) and one in the activation segment (Thr197). These cause important rearrangements in the N-lobe, as demonstrated by changes in the position of the conserved residues K72, E91, and D184. Important rearrangements also occur in the activation- and substrate-binding segments of the C-lobe. The kinase is now catalytically competent and active. The catalytic residue (D166 in the cleft) is indicated by an asterisk (asp*). One of PKA's substrates is the regulatory subunit RIα, and its binding prevents catalytic activity. RIα carries a short inhibitory sequence, -RRGAI- (shown in red and indicated by "IS"), which binds the catalytic cleft but cannot be phosphorylated (the serine is substituted by alanine). Binding of two cAMPs to RIα causes its detachment, allowing the protein kinase to express its activity. The insert shows a surface representation of the PKA holoenzyme, comprising two catalytic and two regulatory subunits. The complex is held together by the dimerization domains (D/D) of RIα. The two D/D domains bind AKAP proteins and these localize the holoenzyme at specific subcellular compartments. Pdb: 1hck,1atp, 2qcs.

subunit, this either is a real substrate (type RII, which can be phosphorylated) or a pseudo-substrate (type RI, which cannot be phosphorylated). The inhibitory sequence (IS) within the regulatory subunit fulfills the linear requirements with respect to the two arginines that must precede the phosphoresidue. For the pseudosubstrate the serine is replaced by alanine, giving rise to-RRGAI- and -RRGGV- for RIα and -β, respectively, and for the real substrate the sequence is -RRVSV- and -RRASV- for RIIα and -β, respectively (return to Figure 2-41(a)). The PKA "holoenzyme" comprises two catalytic subunits that are held together by a dimer of regulatory subunits. The inhibitory constraint is lifted when cAMP binds the regulatory subunits and modifies their shape thereby causing detachment from the catalytic domains. The kinase is now fully active for as long as cAMP levels remain elevated. Another regulatory mechanism involves the N-terminal sequence which, like the C-terminus, wraps around the protein kinase and influence organization of the N-lobe, ATP binding as well as interaction with other proteins (not shown). Moreover, PKA has a myristate bound to the C-terminus which may anchor the protein to the membrane. For review: Taylor et al. (2012a) (Figure 2-43).

A number of cytosolic tyrosine protein kinases, such as Src, Lyn, or c-Abl, need at least two modifications in order to fully express their activity. These protein kinases are composed of multiple domains of which the SH2 and SH3 can be packed against the back of the catalytic domain. In the inactive state of the protein kinase, these accessory domains prevent the correct folding necessary for activity. Moreover, the activation segment needs to be phosphorylated. In the example of c-Abl, when the C-terminal myristate is inserted into the catalytic domain, its SH3 and SH2 are tightly packed and distort the position of the αC-helix. The SH3 domain is fixed to the hinge region between the kinase- and SH2-domains by two prolines (P249 and P242). The protein kinase is in a ″latched and clamped″ (inactive) state. Removal of the myristate, by a myristate-binding protein, causes the kinase to relax in a nearly active conformation (unlatched state). A phosphorylation of the activation segment, on tyrosine-412, follows and this causes it to swing outward. Activity is still augmented when the proline-containing hinge region is phosphorylated on tyrosine-245. This fully prevents attachment of the SH3 domain, leaving the kinase without any constraints—unlatched and unclamped state (return to Figure 2-33(b)) (Nagar et al., 2003, 2006).

The third example concerns an activation process that is quite distinct from many other protein kinases. It deals with the serine/threonine protein kinase-domain in the cytoplasmic segment of the type-1 receptor for TGFβ. The intracellular kinase domain classifies as tyrosine PKL, it has sequence homology with tyrosine kinases but experience showed that it phosphorylates serine and threonine residues. The activation segment is structured but the N-lobe is distorted. This is caused by a glycine-serine-rich domain (GS) that wedges into the N-lobe, held against the αC-helix, and thus prevents the correct positioning of ATP. Phosphorylation of four serine residues in the GS-domain causes its displacement, allowing the αC-helix to switch to its active position. Phosphorylation of GS occurs by the type-II receptor of TGFβ, which is brought into vicinity of the type-I receptor through TGFβ-mediated receptor dimerization (Figure 2-44(a)).

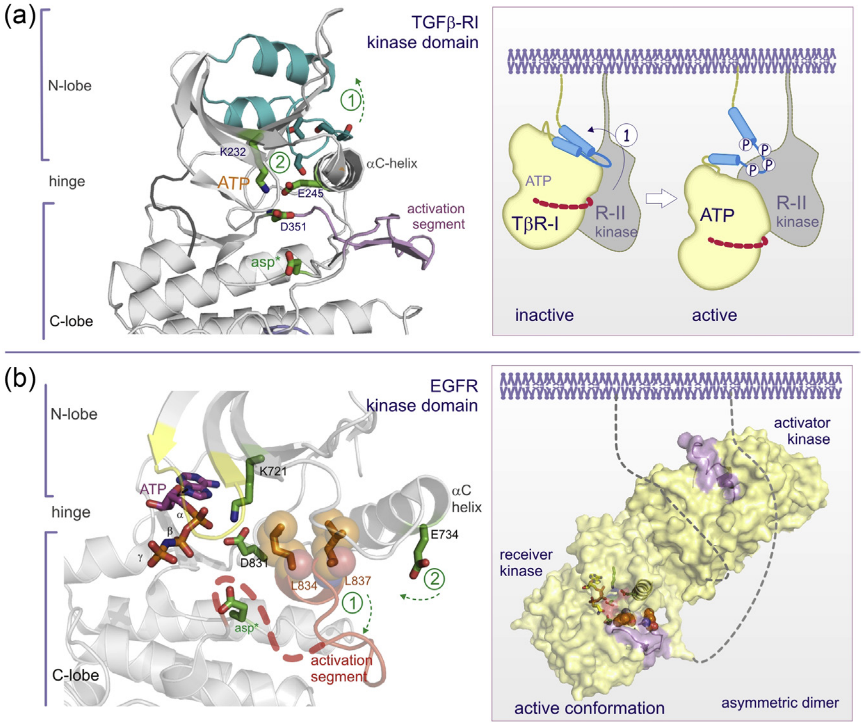


FIGURE 2-44 Activation mechanisms of the EGF and TGFβ growth factor receptors. Both receptors suffer from wedges that distort the N-lobe and thus prevent the correct coordination of ATP. (a) The growth factor TGFβ binds two receptors, type I and type II. Type I (TβR-1) signals into the cell but is inactive, type II is active but only serves to activate type-I. The TβR-1 carries a glycine- and serine-rich domain (GS domain) which is situated on top of the N-lobe and it distorts the N-lobe as indicated by the positioning of two conserved residues, K232 and E245. Upon binding of the ligand, the type II receptor phosphorylates the GS-domain and this causes its detachment and signifies the activation of the receptor kinase domain, (b) The growth factor EGF also binds two receptors, both of which carry inactive kinase domains. Two leucines, which are part of the activation segment wedge into the N-lobe and fully distort the position of the αC-helix (swung-out). Receptor dimerization causes the formation of an asymmetric dimer, where the C-lobe of one kinase (the activator) pushes onto the N-lobe of the other (the receiver kinase). This pushes the leucine wedge away from the N-lobe and allows the αC-helix swing inward, with the conserved E734 now pointing to Mg2+/ATP. Pdb: 1b6c, 2gs7, 2gs6.

As a last example, the tyrosine kinase domain of the EGF receptor does not require any phosphorylation. It is rendered competent through an asymmetric interaction, head to tail, between two kinase-domains of two EGF receptors, in which the binding of one kinase domain (denoted as the "activator kinase") stabilizes the active conformation of the second kinase domain (denoted as the "receiver kinase") (Figure 2-44(b)). The dimerization interface is largely hydrophobic and involves the bottom of the C-lobe of the activator kinase, which docks on the top of the N-lobe of the receiver kinase. The principal interactions in the asymmetric dimer are between helix αH of the activator kinase and helix αC of the receiver. This interaction stabilizes the swung-in conformation of helix αC in the receiver kinase and the extended (swung-out) conformation of the activation loop. The conformational changes that accompany activation and the character and location of the interface within the dimer are both reminiscent of the mechanism of CDK activation by cyclin binding (reviewed in Lowery et al. (2005)). However, the kinase-domain dimer is not stable, and at least in vitro dimer formation requires the cytoplasmic sequence between the membrane and the start of the kinase domain (called juxtamembrane sequence). It is postulated that when the extracellular domains form a stable dimer, in which both receptors are bound to EGF, the juxtamembrane sequences provide an additional interaction between the receiver kinase, which extends its C-terminal juxtamembrane segment (denoted the juxtamembrane latch) to interact with the C-lobe of the activator kinase (not shown) (Zhang et al., 2006; Jura et al., 2009); reviewed in Jura et al. (2011), Red Brewer et al. (2009), Jura et al. (2009), Monsey et al. (2010), Lu et al. (2010).

Web resources for structure/function analysis

PDB, the protein data bank of the Rutgers University and San Diego Supercomputer Center and Skaggs School of Pharmacy and Pharmaceutical Science, is the single worldwide repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids. These are the molecules of life that are found in all organisms including bacteria, yeast, plants, flies, other animals, and humans. http://www.rcsb.org/pdb/home/home.do.

SGC, the Structural Genomics Consortium, is a not-for-profit, public-private partnership with the directive to carry out basic science of relevance to drug discovery. The core mandate of the SGC is to determine 3D structures on a large scale and to cost-effectively target human proteins of biomedical importance and proteins from human parasites that represent potential drug targets. http://www.thesgc.org/scientists/resources/kinases.

PROTEIN PHOSPHATASES

Phosphorylation of proteins serves multiple roles in the regulation of cell function. But this is only half the story. If the transfer of phosphate groups to proteins is to serve as a precise and sensitive signaling mechanism then necessarily it must operate against a low background. Dephosphorylation, therefore, is as important as phosphorylation and it follows that the phosphoprotein phosphatases are integral components of the signaling systems operated by the protein kinases. In a number of cases dephosphorylation serves as a true reset button, bringing proteins back to their inactive state, but dephosphorylation can also constitute the activation button. A good example of this is the role of the S/T phosphatase PPP1CA which dephosphorylates glycogen phosphorylase *a*, thereby terminating the breakdown of glycogen. There are other proteins (for example, glycogen synthase, GSK3β, Src, Lck, c-Jun, and NF-AT) that are inactive in their phosphorylated state, only becoming active as a consequence of dephosphorylation. In the case of Lck, a tyrosine protein kinase, activation of the enzyme requires dephosphorylation of a C-terminal tyrosine residue and then phosphorylation of another tyrosine residue, in the activation segment through an autophosphorylation mechanism (Figure 2-45). To become fully active, the transcription factor c-Jun undergoes dephosphorylation of serine and threonine residues near the DNA-binding site and phosphorylation of serines near the N-terminus (Figure 2-45).

Like for protein kinases, the study on protein phosphatases began in the field of glycogen metabolism. Around 1940, Carl and Gerty Cori, in their search for understanding the regulation of blood glucose levels (glycemia), had discovered that glycogen phosphorylase could be purified in two different forms, one termed phosphorylase *a*, being active, the other phosphorylase *b*, being inactive but susceptible to activation by AMP. The Coris had even identified an enzyme that converted the active into an inactive form. They named the enzyme prosthetic group-removing enzyme (PR) because they thought that the activity was due to some prosthetic group, as it occurs in heme-containing enzymes (Cori and Cori, 1945). Only 10 years later when Fischer and Krebs discovered the role of phosphorylation (Fischer and Krebs, 1955) did it become apparent that PR was in fact a serine/threonine protein phosphatase (now encoded as PPP1CA) (Brautigan, 2013). Phosphatase research started slow for two reasons. First, there was so much to discover in the protein kinase field, a frenzy spurred by two major discoveries; around 1968 when it was shown that cAMP controlled activity of protein kinase A (PKA) (Walsh et al., 1968) and, 13 years later, by the finding that the viral oncogene Src constituted a mutated tyrosine protein kinase (Smart et al., 1981). Secondly, it was generally believed that phosphatases would act as "household enzymes" with low specificity and little modulation of activity. Regulation of phosphorylation would

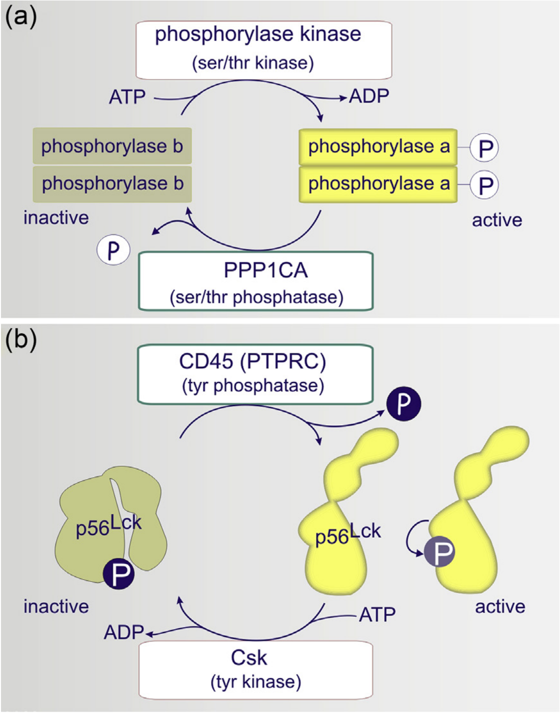


FIGURE 2-45 Inhibitory and stimulatory action of phosphatases. Phosphatases in the generation of signals for both deactivation and activation. (a) Protein phosphatases in the role of a "reset button," deactivating their substrate. An example is the dephosphorylation of phosphorylase-a by the serine phosphatase PPP1CA. (b) Protein phosphatase as activator. CD45, a receptor-like tyrosine protein phosphatase activates, in part, the tyrosine protein kinase p56Lck.

exclusively occur at the level of protein kinases and hence these, and not the phosphatases, were interesting targets for pharmacological intervention in disease (and in particular in cancer).

The held belief was put into question with the discovery, around 1975, of two protein inhibitors of glycogen-phosphorylase phosphatase, named I-1 and I-2. Of these, I-1 was only active when phosphorylated by protein kinase A (PKA) (Huang and Glinsmann, 1976). At the same time new purification procedures had revealed the nature of the glycogen-phosphorylase phosphatase, a 35-kDa protein and it was shown that this protein could associate with regulatory subunits to form higher molecular weight complexes (Brandt et al., 1975). The work of Philip Cohen and coworkers showed that targeting subunits were responsible for the distribution of the 35 kDa-phosphatase into different subcellular fractions and played a role in restricting the substrate specificity. In other words phosphatases too were regulated (Hubbard and Cohen, 1993). Moreover, novel phosphatase assays, the application of inhibitory toxins (for instance okadaic acid), as well the application of cloning techniques, revealed the existence of numerous phosphatases, perhaps not as numerous as protein kinases, but enough to entirely revise the notion that phosphatases were just "housekeeping enzymes."

The finding that certain oncogenes code for tyrosine protein kinases initiated the search for tyrosine phosphatases. Although tyrosine phosphatase activity had been detected in different organs (Gallis et al., 1981), it was Nick Tonks, in the laboratory of Edmond Fischer, who first laid hands on a purified tyrosine phosphatase, extracted from human placenta and named it PTP1B (now recommended as tyrosine-protein phosphatase non-receptor type-1 or PTPN1) (Tonks et al., 1988). Its purification and amino acid sequencing immediately paved the way for a rapid expansion of the subject, starting with the surprise that its amino acid sequence has stretches homologous with the cytoplasmic portion of CD45 (Charbonneau et al., 1988). This protein, a receptor tyrosine phosphatase, is one of the most abundant outer membrane proteins expressed in all nucleated cells of hematopoietic origin and plays a key role in antigen-mediated clonal expansion of T-cell (signaling via the T-cell receptor) (Hermiston et al., 2003). By 1995 the structures of the serine/threonine PPP1CA (Goldberg et al., 1995; Egloff et al., 1995) and of PTP1B (PTPN1) (Barford et al., 1994) had been revealed.

Phosphatase structure and function

In the context of phosphatases involved in signal transduction, we recognize two broad gene families: serine/threonine phosphatases and tyrosine phosphatases. The division in two families corresponds to two different catalytic mechanisms, each employing distinct conserved amino acids and as a consequence distinct 3D structures. The serine/threonine phosphatases are dinuclear metalloenzymes, harboring a combination of Fe^{2+} and Zn^{2+} or $Mg^{2+}$and Mn^{2+}, and they dephosphorylate their substrates in a single reaction step involving a metal-activated nucleophilic-water molecule (or OH–) and a histidine as proton donor (Figure 2-46(a)). It should be noted that for a subfamily of the serine/threonine phosphatases, named PPM, the proton donor still remains elusive (Bellinzoni et al., 2007). The tyrosine phosphatases lack metal ions and catalyze dephosphorylation through a cysteinyl-phosphate intermediate (two-step process) (Figure 2-46(b)). Members of the serine/threonine phosphatases generally do not deal with phosphotyrosine but the reverse is not true, numerous tyrosine phosphatases remove phosphates from phosphoserines and phosphothreonines, they are dual specific. Moreover, roughly 16 members of the tyrosine phosphatases do not even recognize protein; they act on phosphoinositide lipids, mRNA, or glycogen.

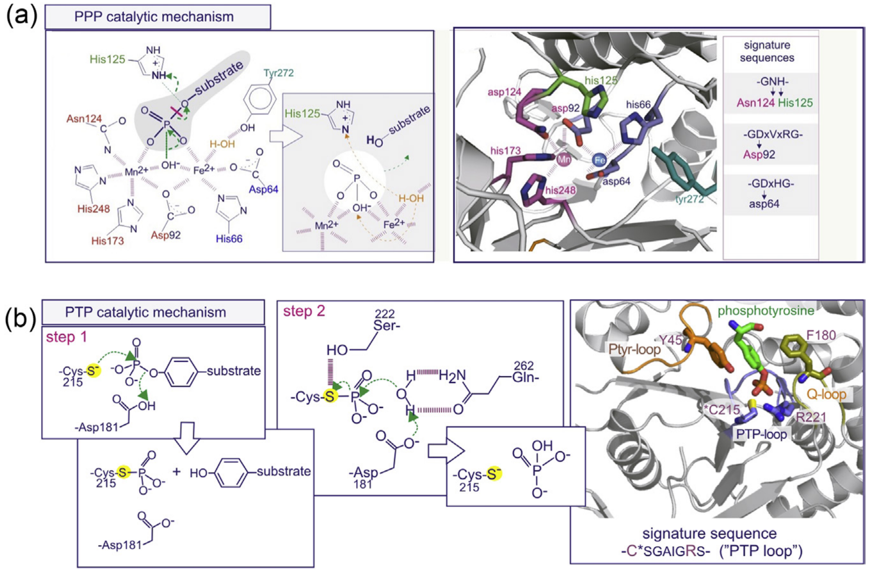


FIGURE 2-46 Molecular mechanism of phosphatase action of the two broad phosphatase families involved in signal transduction. (a) For serine/threonine phosphatases (PPP) the removal of phosphate occurs in one step, where water, or a hydroxide ion, acts as a nucleophile that breaks the bond between phosphate and the substrate. Metal ions play a crucial role in the coordination of water or the hydroxide ion. The metal coordinating residues (asparagine, histidine, and aspartate) are invariant among the PPP family members and they constitute the signature motif. The positions of these highly conserved residues are shown on the right. (b) For tyrosine phosphatases (PTP), in the first step, there is transfer of an electron from C215 to the substrate phosphate. The catalytic cysteine has a low Pka (5.4) so that the sulfhydryl group acts as a nucleophile (–S⁻). This is coupled with protonation of the tyrosyl-leaving group by the side chain of the conserved D181; leading to the formation of a cysteinyl-phosphate intermediate. In the second step, involving Q262 and D181, there is hydrolysis of the catalytic intermediate with release of phosphate. The placement of the essential residues at the active site is depicted on the right. Note that the substrate is sandwiched between Y45 (Ptyr-loop) and F180 (S-loop). These two residues that line the catalytic pocket determine its depth. They are major determinants of specificity for phosphotyrosine. pdf: 1jk7, 1ptt

There exists a third catalytic mechanism, employed by only a small number of phosphatases, in which two aspartates (in a -DxDxT/V- motif) are employed for catalysis, the first serving as the nucleophile and the second as the proton donor. However, these aspartate-based phosphatases are arranged either among the tyrosine phosphatases, members of the EYA family (acting in cytoplasm and nucleus), or among the serine/threonine phosphatases, members of the CTD family (mainly acting in the nucleus). For the record, both EYA and CTD members also fit into the haloacid dehalogenase (HAD) superfamily, a large group of phosphotransferases that target nucleotides, sugars, and proteins as substrates (Thaller et al., 1998).

As for the protein kinases, substrate recognition is multifaceted; factors are the phosphoresidue itself and its adjacent amino acids, subcellular localization and selection either by regulatory subunits associated with the phosphatase or by additional intramolecular domains. Unlike the kinase domain, the phosphatase domain (of both families) has only one lobe. In the case of serine/threonine phosphatases three surface grooves emanate from the active site, which serve binding sites for substrates (and inhibitory toxins), whereas the tyrosine phosphatases form a single shallow substrate pocket. A number of tyrosine phosphatases harbor a secondary-binding pocket and this is thought to accommodate substrates with proximate phosphorylation sites. Unlike the protein kinases, phosphatases are born in a catalytically competent conformation. Regulation by phosphorylation has been reported for a number of protein phosphatases but the phosphoresidues are often outside the catalytic domain and may serve as docking sites for regulatory proteins rather than modifying the structure of the catalytic cleft (as in protein kinases). Tyrosine phosphatases are susceptible to redox regulation; reactive oxygen species can modify the thiolate group of cysteine into a cyclic sulfenamide. With respect to regulation of activity, a subfamily of the serine/threonine phosphatases, named PPP, forms an important exception. They are identified by an unusually high level of sequence conservation and by a complete lack of additional domains (with the exception of PPP5C and PPEF1). As an alternative, these catalytic domains are controlled by regulatory subunits of which some 103 have been identified in man. As we will learn from two examples, these regulatory domains render protein phosphatases specific for a limited number of substrates.

A superfamily of protein phosphatases

There are 155 genes coding for protein phosphatases in the human genome and, as already mentioned, these are separated over two broad groups, serine/threonine (PP) and tyrosine (PTP). Each group constitutes several classes: the serine/threonine group is subdivided into PPP, PPM, and CTD, whereas PTP is subdivided into class I–IV. Each class is made up of one or more families. In addition 103 regulatory proteins are found associated with members of the PPP family and they are classified according to the type of phosphatase they associate with (PPP1R, PPP2R, etc.) (Figure 2-48). Below we briefly deal with the phosphatase families.

PPP family

Members of the PPP family show a remarkable level of sequence identity (around 80%) across species, which is among the highest degree of conservation for any enzyme. They are regarded as very ancient genes, with most members widely distributed in all eukaryotes and most bacterial and archeal genomes having at least one PPP-like member. The PPP family is defined by three signature motifs within their catalytic domain: GDXHG-, -GDXVDRG-, and -GN*HE- (Figure 2-46(a)). With the exception of two members, PPP5C and PPEF, another important characteristic of this family is that they lack additional intramolecular domains but instead increased their diversity by interaction with a large group of regulatory subunits. A number of these regulatory proteins bind a hydrophobic cleft on the phosphatase with a -RVxF/W- amino acid motif (Wakula et al., 2003). Members of PPP1C share 77 regulatory subunits, those of PPP2C share 15, whereas members of PPP3C, PPP4C, and PPPC6 have only few. PPP5C is devoid of subunits but incorporated a TPR domain instead and PFEF has incorporated an EF hand (Ca^{2+}-binding domain) (Moorhead et al., 2009; Brautigan, 2013). Three of the regulatory proteins of PPP1C are also PKA-anchoring proteins (AKAP1, -9, and -11) illustrating once more how kinases and phosphatases are brought together by protein interaction domains (Figure 2-47).

PPM family

The PPM family was identified on the basis of the strict requirement for an exogenous divalent ion for activity (Mg^{2+} or Mn^{2+}), as well as for the insensitivity to known PPP inhibitors such as okadaic acid, microcystin, or calyculin. The catalytic center of PPM resembles that of PPP but the two groups display totally unrelated amino-acid sequences. They are evolved from two unique ancestral genes that converged to highly related structures (Jackson and Denu, 2001). They are identified by nine highly conserved amino acids within a motif that varies from member to member. Four conserved amino acids are aspartate (D) residues that coordinate the metal ions.

CTD phosphatases

They own their name to the fact that they processively dephosphorylate "phosphoSer-2" and "phosphoSer-5" of the heptad repeats -Y**S**PT**S**PS- in the C-terminal domain of the largest RNA polymerase II subunit (hence their name "C-terminal domain" phosphatases). The repeat domain is involved in the initiation of transcription, the capping of the hnRNA transcript, and attachment to the spliceosome for RNA splicing. Phosphorylation creates docking sites for hnRNA processing enzymes (Lima, 2005). The first identified member of CTD phosphatases, back in 1998, was FCP1, so named because it binds to the general transcription factor TFIIA (TFIIF-associated CTD phosphatase) (Archambault et al., 1998). In the new human genome nomenclature FCP1 is termed CTDP1. Apart from the regulation of hnRNA processing, FCP1 is also involved in mitotic exit. Other members of this family control proteasome activity (UBLCP1), membrane-lipid composition (CTDNEP1 and CTDNEP1), or protein transport across the inner mitochondrial membrane (TIMM50).

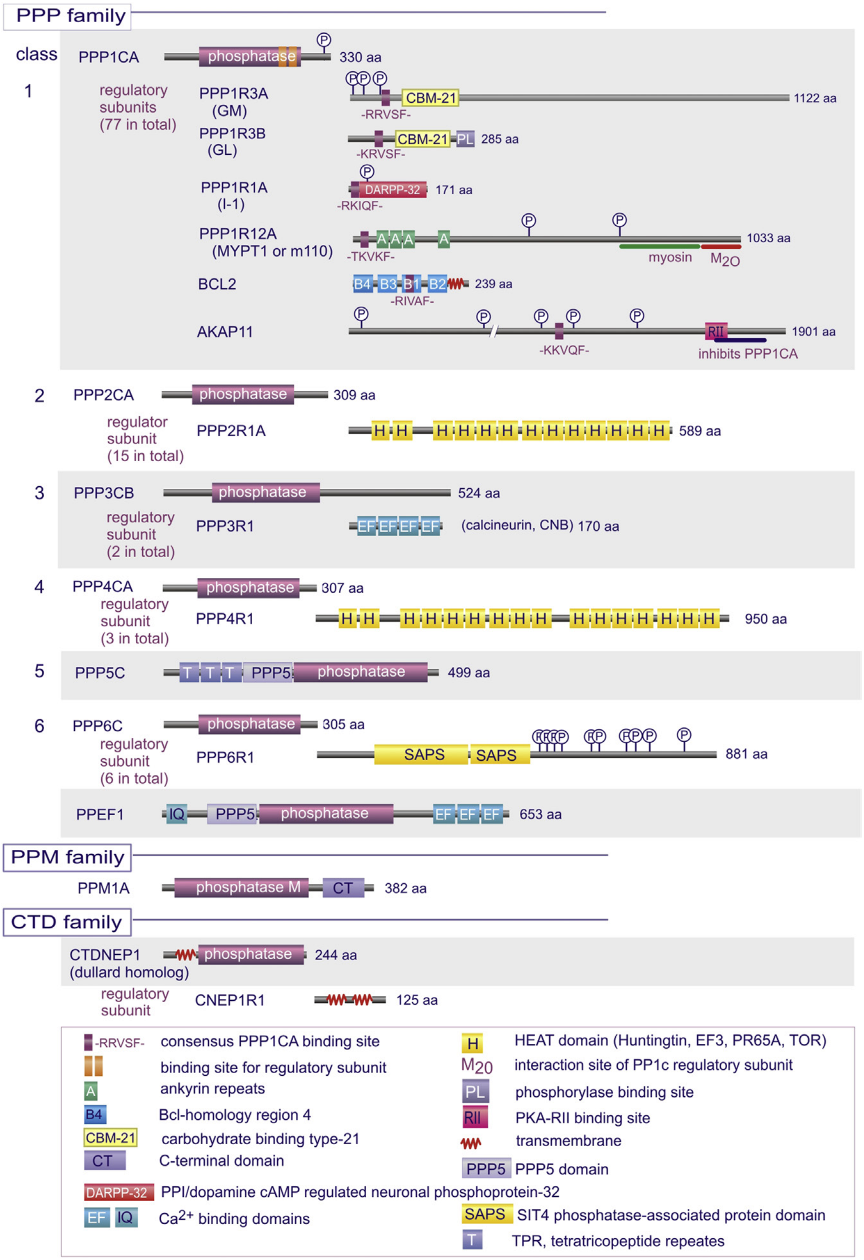

PPP family
class
1
PPP1CA
phosphatase
330 aa
regulatory subunits (77 in total)
PPP1R3A (GM)
CBM-21
-RRVSF-
1122 aa
PPP1R3B (GL)
CBM-21
PL
285 aa
-KRVSF-
PPP1R1A (I-1)
DARPP-32
171 aa
-RKIQF-
PPP1R12A (MYPT1 or m110)
A A A A
-TKVKF-
1033 aa
myosin
M20
BCL2
B4 B3 B1 B2
239 aa
-RIVAF-
AKAP11
-KKVQF-
RII
1901 aa
inhibits PPP1CA
2
PPP2CA
phosphatase
309 aa
regulator subunit (15 in total)
PPP2R1A
H H H H H H H H H H H H H H
589 aa
3
PPP3CB
phosphatase
524 aa
regulatory subunit (2 in total)
PPP3R1
EF EF EF EF
(calcineurin, CNB) 170 aa
4
PPP4CA
phosphatase
307 aa
regulatory subunit (3 in total)
PPP4R1
H H H H H H H H H H H H H H H H H
950 aa
5
PPP5C
T T T PPP5 phosphatase
499 aa
6
PPP6C
phosphatase
305 aa
regulatory subunit (6 in total)
PPP6R1
SAPS SAPS
881 aa
PPEF1
IQ PPP5 phosphatase EF EF EF
653 aa
PPM family
PPM1A
phosphatase M
CT
382 aa
CTD family
CTDNEP1 (dullard homolog)
phosphatase
244 aa
regulatory subunit
CNEP1R1
125 aa
-RRVSF- consensus PPP1CA binding site
binding site for regulatory subunit
A ankyrin repeats
B4 Bcl-homology region 4
CBM-21 carbohydrate binding type-21
CT C-terminal domain
DARPP-32 PPI/dopamine cAMP regulated neuronal phosphoprotein-32
EF IQ Ca2+ binding domains
H HEAT domain (Huntingtin, EF3, PR65A, TOR)
M20 interaction site of PP1c regulatory subunit
PL phosphorylase binding site
RII PKA-RII binding site
transmembrane
PPP5 PPP5 domain
SAPS SIT4 phosphatase-associated protein domain
T TPR, tetratricopeptide repeates

FIGURE 2-47 Domain architecture of members of the PPP family of phosphatases and some of their regulatory subunits. Note that the majority of the phosphatases carry no known protein interaction domains. They interact with regulatory subunits through a specific interaction motif (consensus PPP1CA-binding sites).

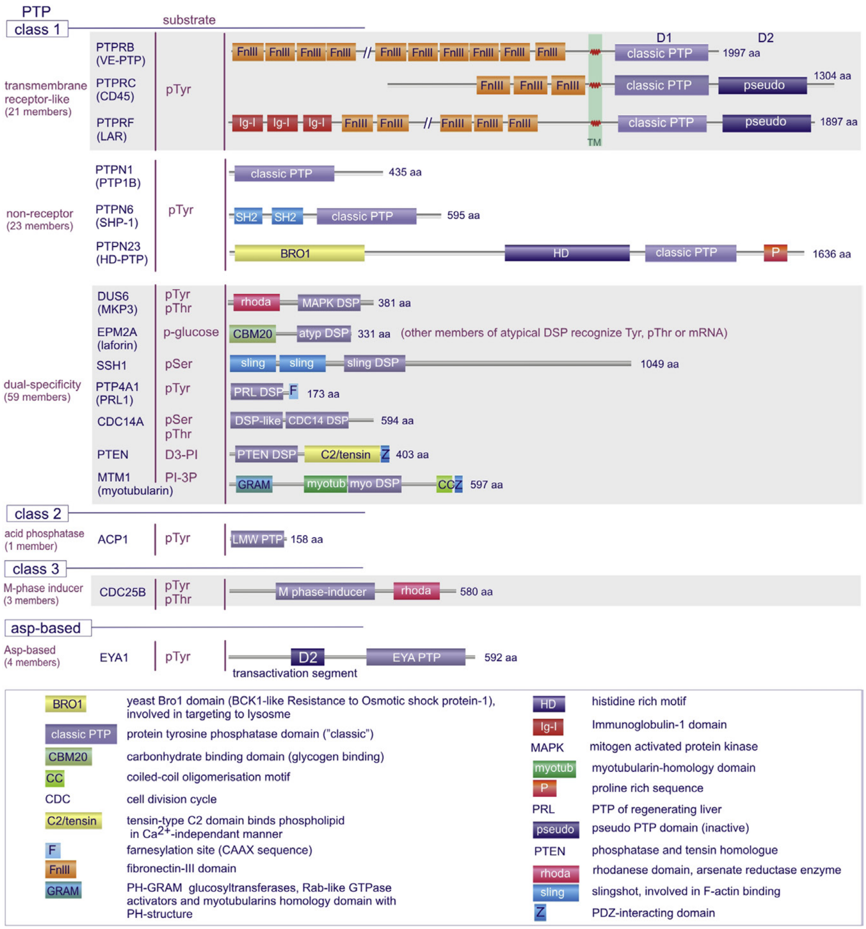

PTP
class 1
substrate
transmembrane receptor-like (21 members)
PTPRB (VE-PTP)
PTPRC (CD45)
PTPRF (LAR)
pTyr
FnIII
D1
D2
classic PTP
1997 aa
1304 aa
pseudo
1897 aa
Ig-I
TM
non-receptor (23 members)
PTPN1 (PTP1B)
PTPN6 (SHP-1)
PTPN23 (HD-PTP)
435 aa
SH2
595 aa
BRO1
HD
P
1636 aa
dual-specificity (59 members)
DUS6 (MKP3)
pTyr pThr
rhoda
MAPK DSP
381 aa
EPM2A (laforin)
p-glucose
CBM20
atyp DSP
331 aa
(other members of atypical DSP recognize Tyr, pThr or mRNA)
SSH1
pSer
sling
sling DSP
1049 aa
PTP4A1 (PRL1)
PRL DSP
F
173 aa
CDC14A
pSer pThr
DSP-like
CDC14 DSP
594 aa
PTEN
D3-PI
PTEN DSP
C2/tensin
Z
403 aa
MTM1 (myotubularin)
PI-3P
GRAM
myotub
myo DSP
CC
597 aa
class 2
acid phosphatase (1 member)
ACP1
LMW PTP
158 aa
class 3
M-phase inducer (3 members)
CDC25B
M phase-inducer
580 aa
asp-based
Asp-based (4 members)
EYA1
EYA PTP
592 aa
transactivation segment
BRO1 yeast Bro1 domain (BCK1-like Resistance to Osmotic shock protein-1), involved in targeting to lysosome
classic PTP protein tyrosine phosphatase domain ("classic")
CBM20 carbonhydrate binding domain (glycogen binding)
CC coiled-coil oligomerisation motif
CDC cell division cycle
C2/tensin tensin-type C2 domain binds phospholipid in Ca2+-independant manner
F farnesylation site (CAAX sequence)
FnIII fibronectin-III domain
GRAM PH-GRAM glucosyltransferases, Rab-like GTPase activators and myotubularins homology domain with PH-structure
HD histidine rich motif
Ig-I Immunoglobulin-1 domain
MAPK mitogen activated protein kinase
myotub myotubularin-homology domain
P proline rich sequence
PRL PTP of regenerating liver
pseudo pseudo PTP domain (inactive)
PTEN phosphatase and tensin homologue
rhoda rhodanese domain, arsenate reductase enzyme
sling slingshot, involved in F-actin binding
Z PDZ-interacting domain

FIGURE 2-48 Domain architecture of members of the PTP family of phosphatases. The tyrosine phosphatases are separated over four groups, each comprising, with the exception of class 2, numerous members. Class one knows three subclasses: transmembrane receptor-like, non-receptor and dual-specificity phosphatases. The dual-specificity phosphatases are further divided in MAPK, atypical, sling, PRL, CDC14, PTEN, and myotubularin phosphatases, again, each having numerous members. Note that not all dephosphorylate pTyr, in particular the atypical DSP and PTEN members have wide-ranging substrates.

PTP family

The PTPs are defined through their common signature motif (C*X5R) that drives catalysis (Figure 2-45(b)). An exception is the aspartate-based class of phosphatases that are accommodated within this family. The human genome contains 107 PTPs. Based on their primary sequence, only 38 of these enzymes appear to be specific for phosphotyrosine, these are named the "classic tyrosine phosphatases." The PTP family is so divergent that they have been further subclassified into four classes of which some are again subdivided into subclasses of enzymes. This diversity likely reflects the richness of the interactions of PTPs with the cell signaling machinery (Tremblay, 2009). PTPs exhibit exceptional substrate specificity in vivo (Tiganis and Bennett, 2007; Alonso et al., 2004).

Class I PTP

This class of protein tyrosine phosphatase contains several families. We thus recognize transmembrane receptor-like phosphatases (PTPR), the non-receptor intracellular phosphatases (PTPN), both families being strictly tyrosine specific (classic PTPs), and the dual-specificity phosphatases (DUSP), which recognize tyrosine and threonine or serine. The majority of transmembrane phosphatases carries two catalytic domains, in tandem, of which only one, the N-terminal or D1, is active. The C-terminal, or D2, is a pseudo-phosphatase, which nevertheless plays a role in regulating the activity of its neighbor. The pseudo-domains are not just inactive D1 mutants; they have distinct amino acid sequences and constitute a subgroup within the PTP family (which, however, is not used for classification). The non-receptor tyrosine phosphatases play a key role in insulin and growth factor signaling. For instance PTPN1 (PTPB1) and PTPN2 (TCPTPT) diminish insulin sensitivity by dephosphorylation of the activation segment of the receptor and of the STAT3 transcription factor (Tiganis, 2013). Curiously, mutational analysis of numerous cancers has revealed loss-of-function mutations for PTPN3, PTPN13, and PTPN14 (Wang et al., 2004), corresponding to their expected role in inhibiting growth factor signaling, but paradoxically, gain-of-function mutations of PTPN11 are associated with leukemia and we now know that PTPN11 is a bona fide oncogene (Mohi and Neel, 2007). Silencing of PTPN21 (PTPD1) was also shown to promote degradation of the EGF receptor and inhibit downstream signaling (rather than stimulate).

The DUSP are a large and very diverse group. They share sequence similarity to the prototypic vaccinia virus phosphatase H1 and are therefore also referred to as VH1-like tyrosine phosphatases (Ishibashi et al., 1992). They are further divided into the MAPK phosphatases (MPKs, involved in growth factor signaling), slingshot phosphatases (dephosphorylate coffilin and this facilitates actin filament assembly), PRL phosphatases (discovered as a phosphatase expressed in regenerating liver, involved cell cycle regulation at the level of progression from G1 to S phase), atypical DUSP (miscellaneous functions which are mostly poorly documented), CDC14 phosphatases (cell division cycle, involved in centrosome separation during mitosis), PTEN phosphatases (phosphoinositide phosphatase involved in growth factor signaling), and MTM phosphatases (myotubularins, phosphoinositotide phosphatases implicated in endocytosis and membrane trafficking, certain mutations lead to myotubular myopathy hence their name). Of this family, 11 members are catalytically inactive and 16 members dephosphorylate either glycogen, mRNA, or phosphoinositides, but not protein.

Class II PTP

This class is represented by only a single member, acid phosphatase (low-molecular-mass PTP or ACP1). We note that it is detected in adipocytes and red blood cells and it expresses alternative splicing variants that give rise to three isoforms. It is otherwise hardly documented.

Class III PTP

This class constitutes three isoforms (A, B, and C) of the cell division-cycle phosphatase CDC25, a key element in the control of cell mitosis. The role of this class of (dual-specificity) phosphatases is to activate protein kinase Cdk1, associated with its regulatory subunit CyclinB. It does so by dephosphorylating two inhibitory phosphoresidues, pT14 and pY15 (Russell and Nurse, 1986). CDC25 itself is subject to numerous phosphorylations, on serine and threonine residues surrounding the phosphatase domain (also known as the“rhodanese domain”). Fifteen sites have been listed which are targets of protein kinases involved in cell cycle regulation such as CDK1, PLK3, CHEK1, BRSK1, and MAPK14 kinases. They are also the substrate of the serine/threonine phosphatase PPP2AC but only when the phosphatase is complexed to the regulatory subunit PPP2R2A. Lee Hartwell, Tim Hunt, and Paul Nurse were rewarded with the Nobel Prize for Medicine in 2001 for their discovery of these cell cycle regulators (Check www.nobelprize.org, laureates 2001, for more information). It is curious that a unique type of PTP evolved to serve regulation of the cell cycle, instead of using members of class I or class II enzymes which presumably already existed at the time. It is possible that an ancestral CDC25 already acted as a rhodanese-type enzyme (catalyzing a sulfur-transfer reaction) on CDKs and was later transformed into a phosphatase when CDK tyrosine/threonine phosphorylation evolved (Alonso et al., 2004).

Class IV PTP

These are members of the aspartate-based protein tyrosine phosphatases and they contain the DxDxT/V motif, identical to the CTD phosphatases that are accommodated in the serine/threonine family. They are all eye-absent homologs (EYA) of which Eya1 was first characterized as a nuclear protein required for eye development in *Drosophila* (Bonini et al., 1993). Ten years later it was shown to be the prototype for a novel family of eukaryotic protein tyrosine phosphatases, placing it in the unusual position of operating as both a transcription factor and an enzyme (Li et al., 2003; Jemc and Rebay, 2007).

Web resource human phosphatase

A rich and very systematic resource of the families, groups, and members of the human phosphatases involved in signal transduction can be found on "gene names," a Web resource curated by the HUGO Gene Nomenclature Committee http://www.genenames.org/.

- PPP phosphatases and their regulatory subunits are listed in: http://www.genenames.org/genefamilies/PPP-PPM-CTD.
- PTP phosphates are listed in: http://www.genenames.org/genefamilies/PTP.

PPP1R12A (MYPT1) AS AN EXAMPLE OF HOW A REGULATORY SUBUNIT CONTROLS SUBSTRATE SELECTIVITY (OF PP1CC)

Smooth muscle contraction, induced by acetylcholine through the muscarinic M3 receptor, starts with the release of Ca^{2+} from intracellular stores. Ca^{2+} binds calmodulin that is complexed with myosin light chain kinase. This leads to activation of the kinase and subsequent phosphorylation of regulatory myosin light-chain at serine-19. Phosphorylation enables the onset of the ATPase cycle and sets in train the paddling of myosin-heads along an actin filament (a cycle of repeatedly binding, ratcheting, and letting go, sliding the thick filament over the thin filament). Reducing the intracellular free Ca^{2+} concentration inactivates the kinase but does not necessarily stop smooth muscle contraction since the myosin light chain remains phosphorylated. Dephosphorylation of the myosin light chain occurs through the myosin-regulatory light-chain phosphatase which is composed of a regulatory subunit PPP1R12A, also known as MYPT-1 or M_{110}, and a catalytic subunit PPP1CC. PPP1R12A serves two purposes: it attaches the phosphatase to myosin, adjacent to the substrate, and it renders it highly selective for the myosin-regulatory light chain. The main outcome of the interaction of PPP1R12A with PPP1CC is the formation of an extended acidic groove, well adapted to accommodate the basic N-terminal sequence of myosin-regulatory light chain and making it much less attractive for other substrates (Figure 2-49) (Terrak et al., 2004).

PPP1CC is a target of okadaic acid, a causative toxin in shellfish poisoning in Europe. Structural analysis revealed that okadaic acid occupies all branches of the Y-shaped catalytic cleft (Maynes et al., 2001). The toxin is produced by dinoflagellates and enters the food chain mainly through bivalve mollusks. "Oka Test," a colorimetric assay for the determination

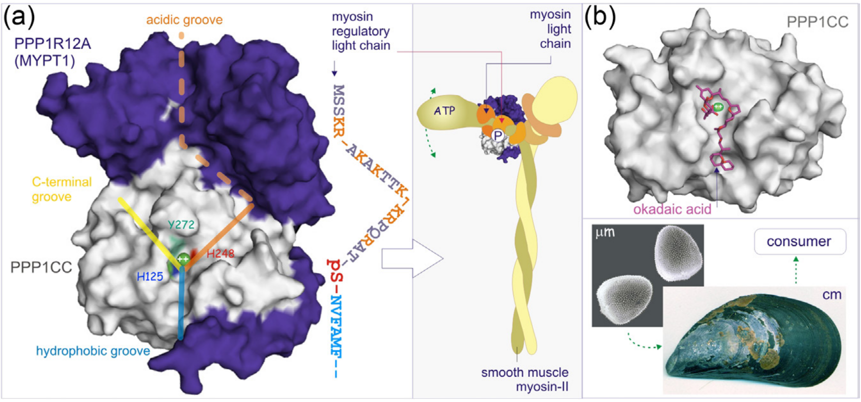


FIGURE 2-49 Regulation of PPP1CC by MYPT1. (a) The surface presentation of PPP1CC shows a shallow Y-shaped catalytic cleft. The three branches of the cleft are commonly referred to as the hydrophobic, C-terminal, and acidic grooves. Substrate could fit into these grooves in different ways. Essential residues are highlighted for orientation purposes; H126 in blue/green, H248 in red, and Y272 in cyan. The catalytic pocket is indicated by a green sphere. Attachment of the regulatory subunit PPP1R12A (MYPT1) to PPP1CC creates an extended acidic groove which perfectly fits the N-terminal sequence of myosin-regulatory light chain (myosin RLC) which holds many basic residues (K or R in orange). Moreover, a stretch of hydrophobic residues (in blue) preferably aligns the hydrophobic groove, positioning phosphoserine right on top of the catalytic pocket. This explains how MYPT increases the affinity (10×) of PPP1CC for this particular substrate. The middle panel shows the location of the myosin RLC on myosin-II. Phosphorylation somehow sets in train the ATP-hydrolysis cycle that is required for head movement and repetitive actin binding, (b) Okadaic acid, a causative toxin of diarrhetic shellfish poisoning in Europe, inhibits PPP1CC by occupying the catalytic cleft thereby preventing access of substrate. The toxin is produced by dinoflagellates and enters the food chain mainly through bivalve mollusks. Pdf: 1s70, 1jk7.

of okadaic acid in shellfish samples employs the activity of the serine/threonine phosphatase PPP2CA as a marker for contamination (Smienk et al., 2012).

REGULATION BY INTRAMOLECULAR DOMAIN INTERACTION, THE EXAMPLE OF PTPN6 (SHP-1)

A role for PTPN6, better known as SHP-1 (SH2 domain-containing phosphatase), was first indicated in mice having a somewhat motheaten appearance with patches of inflamed skin and loss of hair (Tsui et al., 1993). This phenotype, arising from the absence of PTPN6, is due to a systemic autoimmune condition caused by abnormalities in cells of the hematopoietic lineage and it is marked by a general overexpression of cell numbers. The differentiation and functions of natural killer (NK) cells and erythroid cells are also adversely affected. There is an accumulation of macrophages

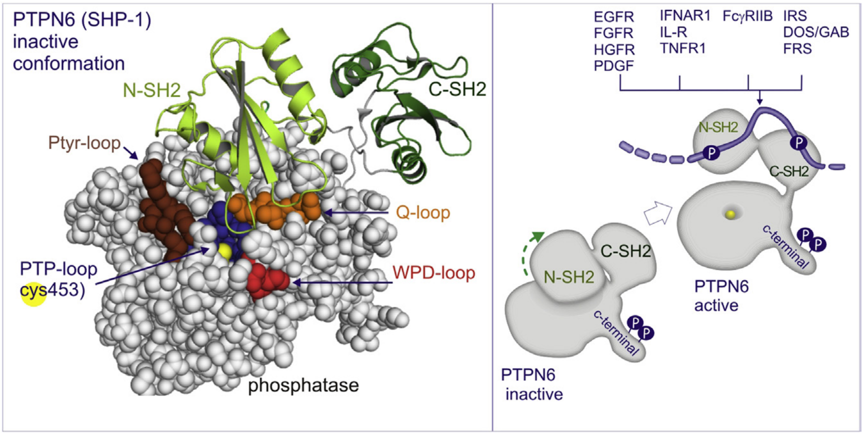


FIGURE 2-50 Regulation of SHP-1 phosphatase (PTPN6) by its intermolecular domains. PTPN6 is a multidomain phosphatase comprising two type-2 Src-homology domains (SH2). The N-terminal SH2 domain folds onto the catalytic pocket and hinders access of substrate. SH2 domains bind phosphotyrosine residues within the context of a short peptide sequence. By engaging phosphotyrosines the SH2-domains swing away and render the phosphatase active. Known tyrosine phosphorylated protein that engage PTPN6 domains are shown on the right (growth factor receptors like EGFR, cytokine receptors like IFNAR1, immune-inhibitory receptors such as FcγRIIB, and docking proteins such as IRS. Pdb: 2b3o.

and neutrophils in the lungs and the mice die within weeks of birth. On this basis, PTPN6 can be regarded as a negative regulator of signaling. Interestingly, PTPN6 expression increases with age in NK cells, rendering the cells less effective killers or more “tolerant” (Chen et al., 2013).

Biochemical and functional studies established the inhibitory role of PTPN6 in a wide range of signaling pathways downstream of receptors for ligands like EPO, prolactin, CSF-1, EGC, BCR, and TCR. There are multiple ways in which PTPN6 interferes, but the common theme is that the phosphatase is invariably recruited into signaling complexes through its SH2 domains (which recognizes phosphotyrosine within the context of short stretch of amino acids). Recruitment into the complex not only serves as a substrate-selection mechanism but also causes the activation of the phosphatase. (Figure 2-50) Normally, the N-terminal SH2 domain wedges into the catalytic cleft of the tyrosine catalytic domain and by binding to the tyrosine phosphorylated receptors, or adaptor proteins in the case of NK cells, removes this inhibitory constraint. In NK cells, SHP-1 dephosphorylates and inactivates the GTP-exchange factor Vav and dephosphorylates the adaptor protein SLP-76, which recruits the MAP-kinase pathway components, whereas the tyrosine kinases Lck, Fyn, and ZAP70 are targets in T-cells (Chen et al., 2013). In neutrophils the Tyk2, Lyn, and PI3-kinase are targets and their dephosphorylation and inactivation render the cells more sensitive to apoptosis (control of innate immunity).

Web resources tyrosine protein phosphatases

- Protein tyrosine phosphatases (including dual-specific) http://www.genenames.org/genefamilies/PTP.
- For supplementary information, see also the resource of Information on protein tyrosine phosphatases (NovoNordisk and Cold Spring Harbor Laboratory) at: http://ptp.cshl.edu or the parallel site http://science.novonordisk.com/ptp).

DECISION-MAKING IN GLYCOGEN SYNTHESIS AND BREAKDOWN: CONCERTED ACTION OF KINASES AND PHOSPHATASES

Below, in a precise example of the regulation of glycogen metabolism, we show the intertwined functioning of protein kinases and phosphatases, both serine/threonine and tyrosine, acting downstream of the insulin and β2-adrenergic receptors in skeletal muscle. Muscles build up a stock of glycogen in the form of particles that are visible under the electronic microscope. Glycogen storage is limited to roughly 230 g in our muscles and this stock is depleted upon intense muscle labor. Insulin plays an important role in glycogen build-up, whereas, as we already have seen, adrenaline stimulates its breakdown. The enzymes involved in glycogen build-up, glycogen synthase, and breakdown,—glycogen phosphorylases—are associated with glycogen particles through docking proteins. For muscle two are important, PPP1R3A (glycogen targeting in muscle, Gm) and PPP1R3C (protein targeting to glycogen, PTG). Glycogen synthase is active when dephosphorylated whereas glycogen phosphorylase is active when phosphorylated (Figure 2-51). Insulin causes dephosphorylation of both, primarily by inhibiting the protein kinases that target glycogen synthase and phosphorylase kinase, whereas adrenaline acts primarily by activating the protein kinase that target glycogen phosphorylase and by inactivating the protein phosphatase that normally keeps glycogen synthase and glycogen phosphorylase in a dephosphorylated state. Starting with insulin, it is released from the pancreas when blood glucose levels rise above 5 mM and it causes activation of the tyrosine protein kinase in the intracellular segment upon binding to its receptor. As a consequence the insulin receptor phosphorylates insulin-receptor substrate-1 (IRS-1), a protein without any known catalytic activity but which serves to recruit enzymes to the plasma membrane (a docking protein). One of the enzymes is the P110 phosphoinositol kinase-alpha (p110α PI3-kinase or PIK3CA), which classifies as an atypical protein kinase and it phosphorylates plasma membrane phosphoinositide lipids rather than protein. The phosphorylated

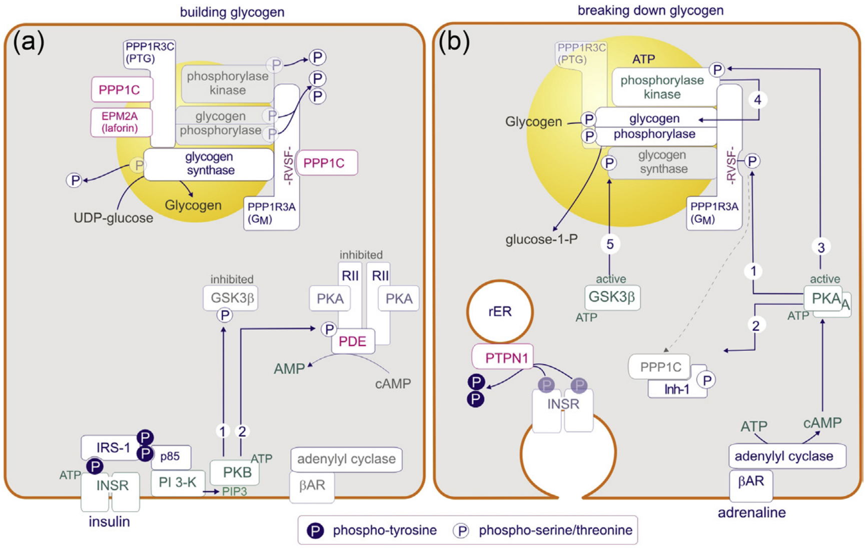


FIGURE 2-51 Decision making in glycogen synthesis and breakdown: opposing effects of adrenaline and insulin. (a) Insulin stimulates glycogen synthesis. This is achieved by silencing two protein kinases. Via the action of PKB, it causes phosphorylation and inactivation of GSK3β (1). PKB also phosphorylates and activates phosphodiesterase (PDE) so depleting cAMP. With both GSK3β and PKA turned off, PPP1c now dominates the scene. It dephosphorylates and inactivates both phosphorylase kinase and glycogen phosphorylase and it activates glycogen synthase. The balance shifts toward glycogen synthesis (incorporation of glucose). (b) Adrenaline drives glycogenolysis through the activation of PKA. It causes the phosphorylation of PPP1R3A (GM) in the PPP1C-binding motif (-RVSF-), causing the two to dissociate. (1). Active PKA also leads to phosphorylation of I-1 which, through the creation of a pseudo-substrate site, more effectively sequesters free PPP1c (2). Finally, PKA phosphorylates and activates glycogen phosphorylase kinase (3) which, in turn, phosphorylates and activates glycogen phosphorylase (4). With the phosphorylation and inhibition of glycogen synthase by GSK3β (5), the balance shifts toward glycogenolysis with liberation of glucose-1-P. The action of insulin is attenuated by the cytoplasmic tyrosine phosphatase (PTPN1). It is attached to the membrane of the rER and dephosphorylates and inactivates the insulin receptor upon its internalization.

inositol-ring (phosphatidyl inositol-3,4-P2) acts as a recruiting site for the serine/threonine protein kinase B-alpha (PKBα or AKT1). This protein kinase, in turn, phosphorylates and stimulates cAMP-phosphodiesterase, an enzyme that breaks the diester bond in cAMP and turns it into AMP. Its action causes a loss of the second-messenger cAMP leading to the inactivation of PKA by its regulatory subunit RI. The second target of PKBα is glycogen synthase kinase-3β (GSK-3β), which is phosphorylated and inactivated (return to Figure 2-33). The loss of activity of both PKA and GSK3β shifts the balance from kinase-dominant to phosphatase-dominant and this leads to dephosphorylation and activation of glycogen synthase and dephosphorylation and inactivation of glycogen phosphorylase. The loss of PKA-activity also causes the return of the phosphatase PPP1C to PPP1R3A (Gm), pushing the equilibrium further toward dephosphorylation. With respect to the role of the docking proteins, expression levels of PPP1R3C (PTG) have profound effects on insulin-mediated glycogen synthesis, whereas PPP1R3A (Gm) has only little impact (McBride and Hardie, 2009).

Adrenaline, as we already have discussed, binds the β2-adrenergic receptor on skeletal muscle, leading to the exchange of GDP for GTP on Gsα with subsequent activation of adenylyl cyclase (type 5) and formation of cAMP. Activated PKA phosphorylates and activates phosphorylase kinase which in turn phosphorylates and activates glycogen phosphorylase (glycogen breakdown). PKA also phosphorylates PPP1R3A (Gm) on residue Ser-65, precisely in the sequence -RVSF- that normally binds PPP1C and as a consequence destabilizes the complex by a factor of 10^4. Moreover, PKA phosphorylates inhibitory protein-1 (I-1) and through this it creates a pseudo-substrate-binding site. As a result, the liberated PPP1C is hidden, unable to dephosphorylate proteins associated with glycogen. The docking protein/regulatory subunit PPP1R3C (PTG) lacks the serine in the PPP1C-binding motif, which is -RVVF- and as a consequence PKA cannot destabilize the interaction. Regulation of phosphatase activity remains to be clarified. The action of insulin is counteracted by PTPN1 (PTP1B), a phosphatase that removes tyrosine residues from the insulin receptor activation segment. Loss of this phosphatase, by gene knockout, makes cells extremely sensitive to insulin signaling, thus proving its importance as a negative feedback regulator (Salmeen et al., 2000; Elchebly et al., 1999). Interestingly, PTP1B is anchored to the cytosolic face of the endoplasmic reticulum (ER) via a hydrophobic C-terminal targeting sequence and it dephosphorylates the insulin receptor (IR) only after endocytosis, as it transits past the ER (Haj et al., 2002; Boute et al., 2003).

The docking protein PP1R3C (PTG) merits further exploration (Munro et al., 2005; Printen et al., 1997). Not only does it bind PPP1C (N-terminal region) and glycogen (through its carbohydrate-binding domain, CBM21, in the central region, return to figure 2-47), it also binds glycogen phosphorylase and glycogen synthase with its C-terminal region. Lastly, it binds the atypical dual-specificity phosphatase EPM2A (laforin). EPM2A (epilepsy, progressive myoclonus type 2A) is the only phosphatase with a carbohydrate-binding domain (CBM21) but more importantly, recessive mutants of this protein are associated with teenage-onset neurogenerative epilepsy named Lafora disease. The consequence of the mutation is a poorly branched and water insoluble type of glycogen (polyglucosan) that accumulates in various tissues. In the brain, these accumulations (termed

Lafora bodies) overtake neuronal dendrites and provoke neurodegeneration and intractable and fatal seizures (Minassian et al., 1998). Studies in knockout mice show that lack of the phosphatase results in hyperphosphorylation of glucosyl-C6 carbons and this alters the structure of glycogen. In other words, in the cascade of kinases and phosphatases we can add another level of regulation in the metabolism of glycogen, at the level of glycogen phosphorylation. The C6-glucose kinase is not yet determined (Nitschke et al., 2013).

Finally, in the absence of adrenaline and insulin, the glycogen metabolism seems to be determined by the glycogen content. One of the protein kinases that binds glycogen is AMPK and it phosphorylates (on Ser-8) and inactivates glycogen synthase. The general hypothesis is that in the abundant presence of glycogen, AMPK keeps glycogen synthase in its inactive state. Upon loss of glycogen branches, the kinase and the glycogen synthase are separated and dephosphorylation by PPP1C dominates, leading to glycogen synthesis. AMPK is known as the “energy sensor,” it gets activated when levels of ATP are low, and as a consequence the levels of AMP are high (not to be confounded with cAMP!) (McBride and Hardie, 2009) (Figure 2-51).

We suggest the following review to learn more about the regulation of glycogen metabolism (Roach et al., 2012).

Web resource of signaling pathways

Abundant information about signaling pathways, in the form of schemes can be obtained at Cell Signaling Technology http://www.cellsignal.com/pathways/index.html.

SIGNAL TERMINATION

All signaling pathways have feedback mechanisms, both positive and negative. Positive feedback loops can induce switch-like or bi-stable behavior: the stimulus dose–response curve is not linear, but remains low until a threshold is reached and then sets at full scale (an analogous input is converted into a digital (non or all) output (Ferrel, 2002)).

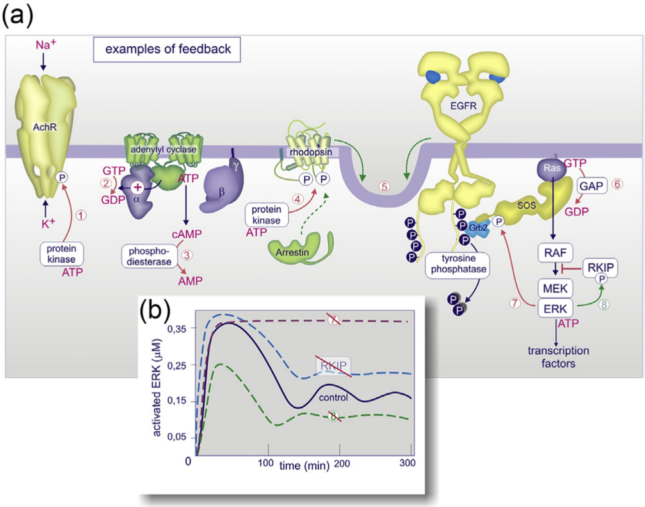


FIGURE 2-52 Feedback mechanisms in signaling pathways. (a) most receptors and downstream signaling components are subject to feedback mechanisms. For instance, the nicotinic acetylcholine receptor becomes refractory upon phosphorylation by a protein kinase (1), the effector adenylyl cyclase promotes the hydrolysis of GTP and thereby terminates production of cAMP (2), the phosphodiesterase turns cyclicAMP into AMP (3), certain protein kinases phosphorylate G-protein-coupled receptors (rhodopsin in this example) and this leads to binding of arrestin which blocks further interaction with heterotrimeric G-proteins (4), many receptors are internalized (5). Finally, GAPs hydrolyze GTP on Ras, thus terminating a growth factor signal (EGF for instance). Further downstream in the EGF signaling cascade, involving the protein kinases (RAF, MEK, and ERK), both positive and negative feedback emerges. The activated protein kinase ERK phosphorylates SOS and this leads to its detachment from Grb2 and subsequent detachment from the membrane. SOS is a GTP exchange protein and activates RAS. ERK also phosphorylates RKIP, a protein that hinders fast activation of MEK by RAF. Phosphorylation of RKIP speeds up the activation cascade. (b) Experimentally derived data from signaling events have been processed into a simulation program of EGF-mediated activation of ERK. This type of approach helps to predict how cells react upon pharmacological intervention in the case of aberrant cell proliferation (as in cancer). The graph shows that despite a permanent presence of growth factor, the cells eventually reduce the signal through negative feedback loops (process 7). Loss of this negative feedback results in a constant elevated signal. Removal of a positive feedback loop (process 8) greatly diminishes the amplitude of the response. Finally, removal of RKIP, which normally is a rate-limiting component in the cascade, both increases the speed and the extent of the growth factor response. *Image (b) is adapted from Shin et al. (2009).*

Negative feedback is crucial for two reasons, it limits the cellular response (system stability) (Kholodenko, 2000) and it makes cells sensitive to new environmental signals. In the section about G-proteins we have given the example of photoreceptors that are immediately desensitized in order to allow a reset of the initial light signal (page 114) (Figure 2-52).

Inhibitory feedback takes place at different levels: the receptor can be modified and made resistant (in particular ligand-controlled ion channels) or it can be removed from the membrane by endocytosis (all receptors). Signaling complexes can be deactivated by dephosphorylation (reaction catalyzed by a phosphatase) or the GTP can be hydrolyzed (reaction catalyzed by GAPs). The receptor can be modified, by phosphorylation, and then binds an arresting component which prevents interaction with the initial, feed-forward, effector proteins. Components of the signaling complex can be modified such that the cascade will be interrupted (example of Grb2/Sos). In certain instances, the signaling pathway will induce expression of not only the genes that feed-forward but also those that terminate the response.

Nearly all immediate feedback mechanisms, occurring in a time span of seconds and not involving gene expression, initially give rise to oscillations before attenuating the signal all together. Oscillations have been observed for the second-messenger Ca^{2+} and cAMP but also for the protein kinases such as PKA or ERK. Oscillating membrane potentials that generate rhythmic impulse patterns are significant for neuronal information and they cause our heart to beat at a steady pace even when it is disconnected from the body.

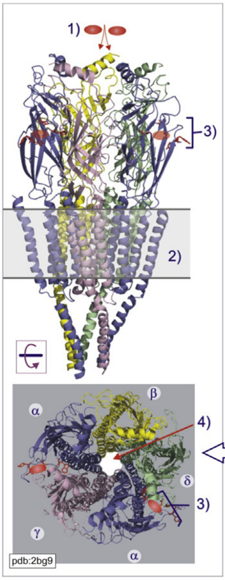
A lack of inhibitory feedback, through gain-of-function mutations in for instance protein kinases or G-proteins, causes inappropriate cellular responses as made manifest in cancer. Insufficient inhibitory feedback is also the cause of numerous inflammatory conditions, where immune cells fail to resolve the inflammatory state and keep spitting out cytokines and chemokines that gradually destroy the tissue. We have given the example of the motheaten phenotype of mice that lack the phosphatase PTPN6 (SHP-1). Complex as they may be, understanding feedback mechanisms is probably essential for the design of pharmacological agents that target signaling pathways.

As an example, to illustrate the subject a little further, we show how growth factor signals are enhanced and then attenuated by a positive and a negative feedback mechanism. We caution that modeling of signaling pathways for complex organisms still is in its infancy, not because of a lack of talented scientists, mathematical, and computational power but because of new components and new interactions that are still discovered and that constantly brings new parameters into the equation. Moreover, cells do not deal with one but with multiple inputs and these, as we have shown, communicate with each other (cross-talk).

The pathway in question initiates at the receptor for the EGF (right section of figure 2-52). Growth factor binding and receptor dimerization lead to activation of the intracellular tyrosine kinase segment which causes reciprocal phosphorylation of the c-terminal tails of the receptors. The phosphotyrosine residues recruit a guanine-nucleotide exchange complex (Grb2/SOS) which exchanges GDP for GTP in the small G-protein Ras. This leads to recruitment, phosphorylation, and activation of Raf. The next step, where Raf phosphorylates and activates MEK, is rate limiting, and subject to positive feedback. Once Raf phosphorylates MEK, the signal is transmitted to ERK which detaches from the complex at the membrane and phosphorylates numerous substrates in the cytoplasm and in the nucleus. Among the substrates is SOS and its phosphorylation lead to its detachment from Grb2 and thus from the membrane. SOS folds into an auto-inhibited state and the Ras-signal attenuates. The other substrate is RKIP, which when phosphorylated is no longer rate limiting, thus speeding up signal transition from Raf to MEK. Based on experiments with real cells, the cascade has been modeled and this allows to predict possible outcomes by varying the parameters. For instance what happens when you block the positive feedback or you remove RKIP altogether. Figure 2-52(b) shows such simulation where the stimulus remains present throughout the simulation (300 min). In the control situation, ERK kinase activity (end of the cascade) peaks at 20 min and then oscillates to a stable lower level. Removal of RKIP, the rate-limiting component, causes a much faster response but the subsequent oscillation remains. Removing the positive feedback loop, which normally acts on RKIP, severely diminishes the response. On the contrary, without negative feedback on SOS the curve remains high throughout the simulation (Shin et al., 2009).

IF VIRTUAL CELLS HAVE YOUR INTEREST WE SUGGEST TO EXPLORE THE SUBJECT BY THE FOLLOWING ARTICLES:

- Freddolino P.L., Tavazoie S., 2012. The dawn of virtual cell biology Cell 150, 248–250.
- Karr J.R., Sanghvi J.C., Macklin D.N., Gutschow M.V., Jacobs J.M., Bolival Jr., B., Assad-Garcia N., Glass J.I., Covert M.W., 2012. A whole-cell computational model predicts phenotype from genotype Cell 150, 389–401.
- Concalves E., Bucher J., Ryll A., Niklas J., Mauch K., Klamt S., Rocha M., Zaez-Rodriquez J., 2013. Bridging the layers: towards integration of signal transduction, regulation and metabolism into mathematical models. Mol. Biosyst. 9, 1576–1583.



ligand-gated ion channels

first messenger and its receptor (in bold)	functions (among others)
- acetylcholine : nicotic receptor **AchRN** (Na$^+$, K$^+$)	-initiation of the action potential
- ATP : purinergic receptor **P2X** (Na$^+$, Ca^{2+})	-change in membrane protential, increase in intracellular Ca^{2+}
- γ-aminobutyric acid : **GABA-R** (Cl$^-$)	-"inhibitory" neurotransmitter
- glutamate : ionotropopic receptor **GluR** (receptors AMPA, NMDA and Kainate)(Na$^+$, K$^+$, Ca^{2+})	-"excitatory" neurotransmitter
- glycine : **GlR** (Cl$^-$)	-"inhibitory" neurotransmitter
- sérotonine (5HT) : **5HTR3** (Na$^+$, K$^+$, Ca^{2+})	-"excitatory" neurotransmitter

canaux ioniques voltage dépendant

- membrane potential : voltage-dependant K$^+$ channel **Kv**	-membrane repolarisation
- potentiel membranaire : voltage-dependent Na$^+$ channel **Nav**	-action potential

molecular structure of the nicotinic acetylcholine receptor (AchRN)

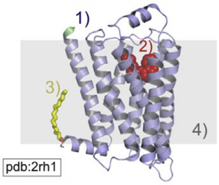
1 acetylcholine (ligand)
2 plasma membrane
3 acetylcholine binding pocket
4 pore of ion channel

α, β, γ and δ are protein subunits that constitute the receptor

FIGURE 2-A Ligand-gated ion channels.

type 2: G-protein coupled receptors (GPCR or 7TM)

first messenger and its receptor (in bold)	functions (among others)
- acetylcholine : **AchRM** (muscarinic)	-contraction of smooth muscle, slow down of cardiac rhythm (Vagus nerve)
- adrenocorticotropic hormone : **ACTHR**	-production of cortisol by the adrenal gland
- adenosine : **P1R (**purinergic receptor)	-relaxation of smooth muscle, arrousel state (brain)
- adrenaline and noradrenaline : **ADR** or **AR**	-glycogenolysis, smooth muscle contraction, increase in force and rhythmicity of the cardiac muscle



adrenergic receptor type-β2 (β2-AR ou ADRB2)

1 receptor
2 carbazolole, ligand, antagonist of adrenaline
3 palmitate (fatty acid, C16, covalently bound to cysteine-341)
4 plasma membrane

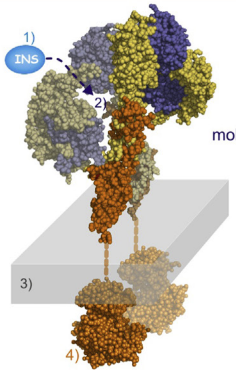
first messenger and its receptor (in bold)	functions (among others)
- angiotensin II : **AGTR**	-secretion of aldosterone, contraction of smooth muscle
- ATP (and UTP) : **P2YR**	-secretion of chemokines, synaptic transmission, etc
- bradykinin : **BdkR**	-inflammation, algesia, secretion of ions in the intestinal mucosa
- calcitonin : **CALCR**	-inhibiting osteoclast functioning
- complement : **C5aR, C3aR**	-chemokine, inflammation
- dopamine : **DR**	-neutrotransmitter involved in neuronal reward circuits, , motor function (deficient dopamine functioning in Parkinson)
- eicosanoides : **PDGR, PDGER, LTBR, TBXAR**	-prostaglandins, leukotrienes, tromboxanes, prostacyclins: inflammation, coagulation
- formyl-met-leu-phe (fMLP) : **FPR**	-bacterial component, chemokine, inflammation
- gamma aminobutyric acid: **mGABA$_B$R** (metabotropic)	-“inhibitory” neurotransmitter
- follicle stimulating hormone : **FSHR**	-maturation of avarian follicles (granolosa cells and oocyte)
- glucagon : **GR**	-glycogenolysis (breakdown of glycogen)
- glutamate (metabotropic : **mGluR**	-”excitatory” neurotransmitter
- photons (light) : **rhodopsine**	-vision
- luteinizing hormone : **LHR**	-induction of ovulation and of ovarian steroid secretion
- interleukin-8 : **IL8R**	-chemokine, inflammation
- lysophosphatidic acid : **LPAR**	-growth factor, neurite retraction
- melanocyte stimulating hormone : **MSHR**	-melanin synthesis (skin pigment)
- neurokinines (substance P, neurokininA, -B): **NKR**	-neuropeptides, anxiety, analgesia, insulin secretion
- odorant molecules : **OR** (at least 400 different receptors)	-olfaction (smell)
- enkephalin, opioids : **OPR**	-neurotransmitter, analgesia, neuronal reward system
- oxytocin : **OTR**	-peptide hormone, smooth muscle contraction of the uterus, milk ejection, maternal bonding
- parathyroid hormone : **PTHR**	-absorption of calcium in the bone (formation of osteoclasts), renal absorption of calcium
- thrombin : **PAR** (protease activated receptor)	-platelet clotting factor (coagulation)
- thyroid stimulating hormone : **TSHR**	-secretion of thyroid hormone
- vasopressin : **AVPR**	-apical membrane expression of aquaporin in the kidney
- wingless : **Fz** (frizzled, phenotype of Drosophile)	-cytokine involved in the development and cell differentiation (morphogen)

FIGURE 2-B G-protein-coupled receptors (GPCR).

receptors coupled to an intrinsic kinase or cyclase activity

tyrosine kinases (growth factor receptors)

first messenger and its receptor (in bold)	functions (among others)
- epidermal growth factor : **EGFR** and **ERBB**	-regulation of cell proliferation and differentiation
- fibroblast growth factor : **FGFR**	-mesoderm formation during development, fibroblast proliferation, cell migration, angiogenesis
- hepatocyte growth factor or scatter factor (SF) : **MET**	-regulation of cell motility, cell proliferation and degradation of extracellular matrix
- insulin : **INSR**	-glucose transport, glycogen synthesis, protein synthesis, cell proliferation.
- insulin-like growth factor : **IGFR**	-critical factor in the growth of the human body
- macrophage colony-stimulating factor : **M-CSFR**	-macrophage differentiation (hematopoietic precursor cells)
- neurotrophins (nerve growth factors) : **NTRK**	-survival, development and functioning of neurons
- platelet-derived growth factor : **PDGFR**	-regulation of cell proliferation, differentiation and migration
- growth arrest-specific 6 (GAS6) : **AXL**	-regulation of cell proliferation, adhesion, survival and platelet aggregation
- ephrin : **EPH**	-regulation of cell differentiation and migration
- vascular endothelial growth factor : **KDR** and **FLT (VEGFR)**	-angiogenesis and vascular permeability
- transforming growth factor alpha (TGFα) : **EGFR** (ERBB1)	-regulation of cell proliferation and differentiation



molecular structure of the insulin receptor (dimer)

1 insulin
2 insuling binding pocket
3 plasma membrane
4 intracellular segment: tyrosine protein kinase

pdb:2dtg + 1irk

serine/threonine kinases

-activin : **ACVR**	-regulation of FSH secretion, morphogenesis, wound healing
-bone morphogenetic protein : **BMPR**	-morphogen, differentiation of osteoblasts
-transforming growth factor beta (TGFβ) : **TGFBR**	-regulation of differentiation, cell migration, matrix deposition, inflammation

guanylyl cyclase

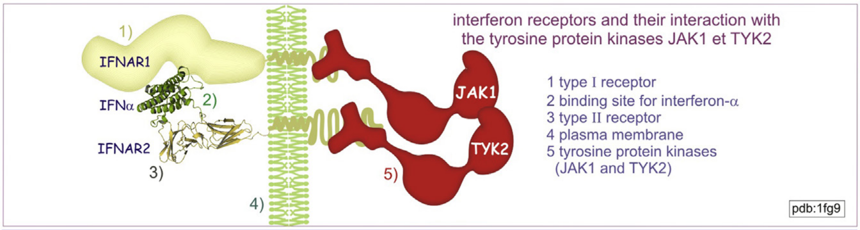
- atrial natriuretic peptide : **NPR**	-reduction of blood pressure (renal excretion of Na^+/H20)

FIGURE 2-C Receptors coupled to an intrinsic kinase or cyclase activity

receptors coupled to a protein kinase

cytokine receptors

first messenger and its receptor (in bold)	functions (among others)
-erythropoetin : **EPOR**	-growth factor for precursors of red blood cells
-prolactin : **PRLR**	-growth of mammary gland, milk secretion
-growth hormone (somatotropin) : **GHR**	-growth factor (direct effect on chondrocytes), induces expression of IGF
-interleukin-2 : **IL-2R**	-inflammation, clonal expansion of T-type lymphocytes
-interferon : **IFN-R**	-immune defense, differentiation of hematopoietic precursor cells
-granulocyte colony-stimulating factor : **CSF-R**	-production of granulocytes and stem cells in bone marrow


interferon receptors and their interaction with
the tyrosine protein kinases JAK1 et TYK2
1)
IFNAR1
IFNα
2)
IFNAR2
3)
4)
5)
JAK1
TYK2
1 type I receptor
2 binding site for interferon-α
3 type II receptor
4 plasma membrane
5 tyrosine protein kinases
(JAK1 and TYK2)
pdb:1fg9

TNF receptors

-tumor necrosis factor-alpha (TNFα) : **TNFRSF**	-innate immune response, apoptosis, cachexia
-FAS ligand (FS-7 cell associated surface antigen : **FAS**	-apoptosis (programmed cell death)

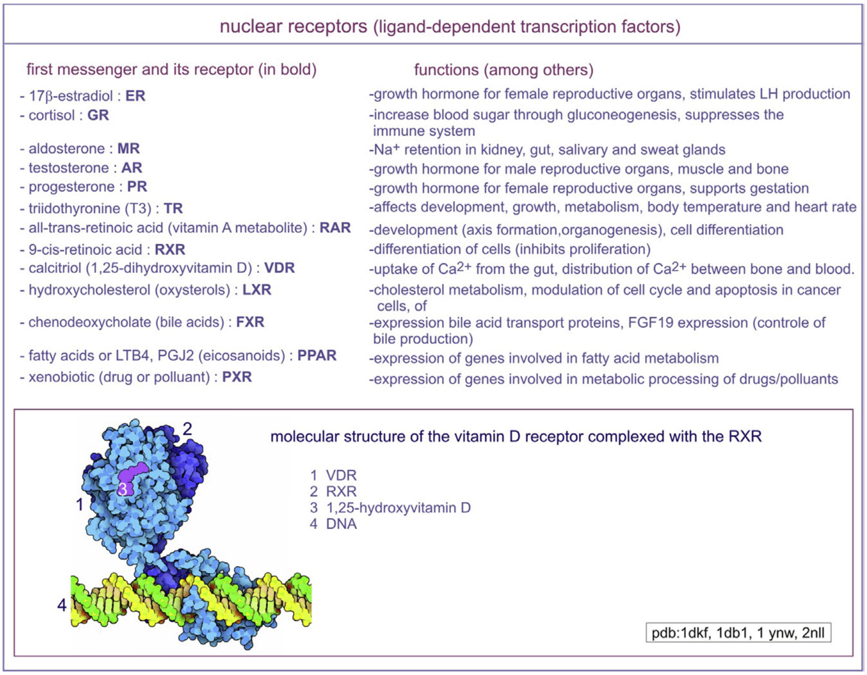
Toll-like receptors (TLR)

-Lipopolysaccharide (LPS) : **TLR4** (toll-like receptor)	-initiation of the innate immune response, inflammation
-interleukin-1 : **IL-1R**	-inflammatory response, fever,
-viral DNA : **TLR3**	-initiation of the innate immune response, inflammation

receptors for antigens (in MHC context) and immunoglobulins (Fc)

-antigen bound to the major histocompatibility complex : **TRA** and **TRB**	-recognition of antigen, initiation of the adaptive immune response
-immunoglobulin-G fixed to antigen : **FCGR1**	-endo-/phagocytosis, activation of type B lymphocytes

FIGURE 2-D Single membrane pass receptors coupled to an extrinsic kinase activity (bound to intracellular segment)



nuclear receptors (ligand-dependent transcription factors)

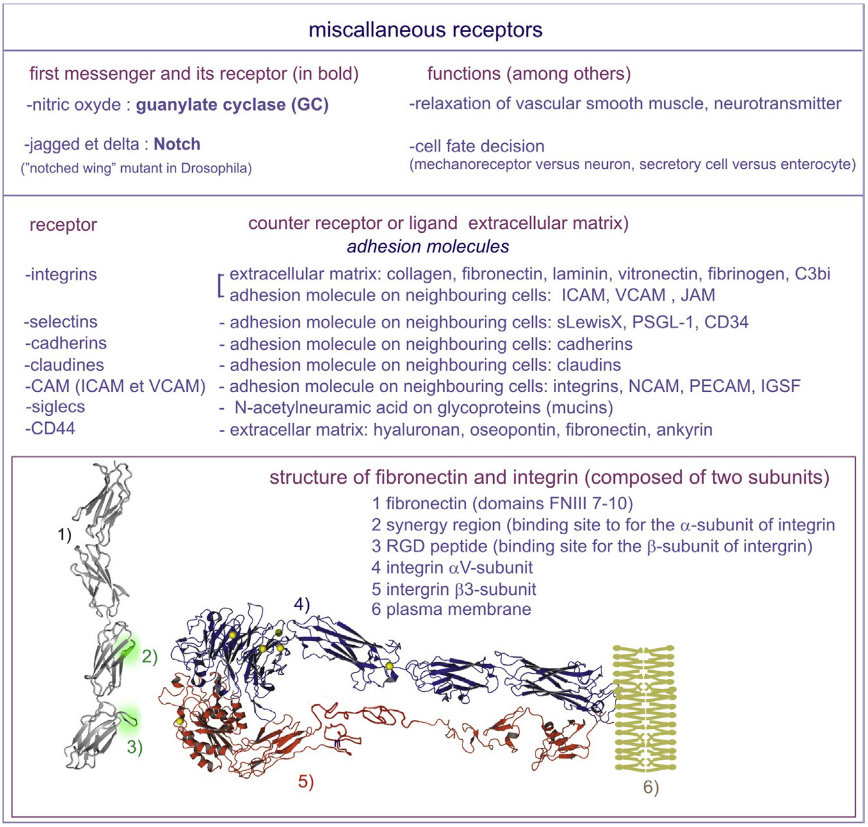
first messenger and its receptor (in bold)	functions (among others)
- 17β-estradiol : **ER**	-growth hormone for female reproductive organs, stimulates LH production
- cortisol : **GR**	-increase blood sugar through gluconeogenesis, suppresses the immune system
- aldosterone : **MR**	-Na+ retention in kidney, gut, salivary and sweat glands
- testosterone : **AR**	-growth hormone for male reproductive organs, muscle and bone
- progesterone : **PR**	-growth hormone for female reproductive organs, supports gestation
- triidothyronine (T3) : **TR**	-affects development, growth, metabolism, body temperature and heart rate
- all-trans-retinoic acid (vitamin A metabolite) : **RAR**	-development (axis formation,organogenesis), cell differentiation
- 9-cis-retinoic acid : **RXR**	-differentiation of cells (inhibits proliferation)
- calcitriol (1,25-dihydroxyvitamin D) : **VDR**	-uptake of Ca^{2+} from the gut, distribution of Ca^{2+} between bone and blood.
- hydroxycholesterol (oxysterols) : **LXR**	-cholesterol metabolism, modulation of cell cycle and apoptosis in cancer cells, of
- chenodeoxycholate (bile acids) : **FXR**	-expression bile acid transport proteins, FGF19 expression (controle of bile production)
- fatty acids or LTB4, PGJ2 (eicosanoids) : **PPAR**	-expression of genes involved in fatty acid metabolism
- xenobiotic (drug or pollutant) : **PXR**	-expression of genes involved in metabolic processing of drugs/polluants

molecular structure of the vitamin D receptor complexed with the RXR

1 VDR
2 RXR
3 1,25-hydroxyvitamin D
4 DNA

pdb:1dkf, 1db1, 1 ynw, 2nll

FIGURE 2-E Nuclear receptors



miscallaneous receptors

first messenger and its receptor (in bold)	functions (among others)
-nitric oxyde : **guanylate cyclase (GC)**	-relaxation of vascular smooth muscle, neurotransmitter
-jagged et delta : **Notch** ("notched wing" mutant in Drosophila)	-cell fate decision (mechanoreceptor versus neuron, secretory cell versus enterocyte)

receptor	counter receptor or ligand extracellular matrix)
	adhesion molecules
-integrins	extracellular matrix: collagen, fibronectin, laminin, vitronectin, fibrinogen, C3bi adhesion molecule on neighbouring cells: ICAM, VCAM , JAM
-selectins	- adhesion molecule on neighbouring cells: sLewisX, PSGL-1, CD34
-cadherins	- adhesion molecule on neighbouring cells: cadherins
-claudines	- adhesion molecule on neighbouring cells: claudins
-CAM (ICAM et VCAM)	- adhesion molecule on neighbouring cells: integrins, NCAM, PECAM, IGSF
-siglecs	- N-acetylneuramic acid on glycoproteins (mucins)
-CD44	- extracellar matrix: hyaluronan, oseopontin, fibronectin, ankyrin

structure of fibronectin and integrin (composed of two subunits)

1 fibronectin (domains FNIII 7-10)
2 synergy region (binding site to for the α-subunit of integrin
3 RGD peptide (binding site for the β-subunit of intergrin)
4 integrin αV-subunit
5 intergrin β3-subunit
6 plasma membrane

FIGURE 2-F Miscallaneous receptors

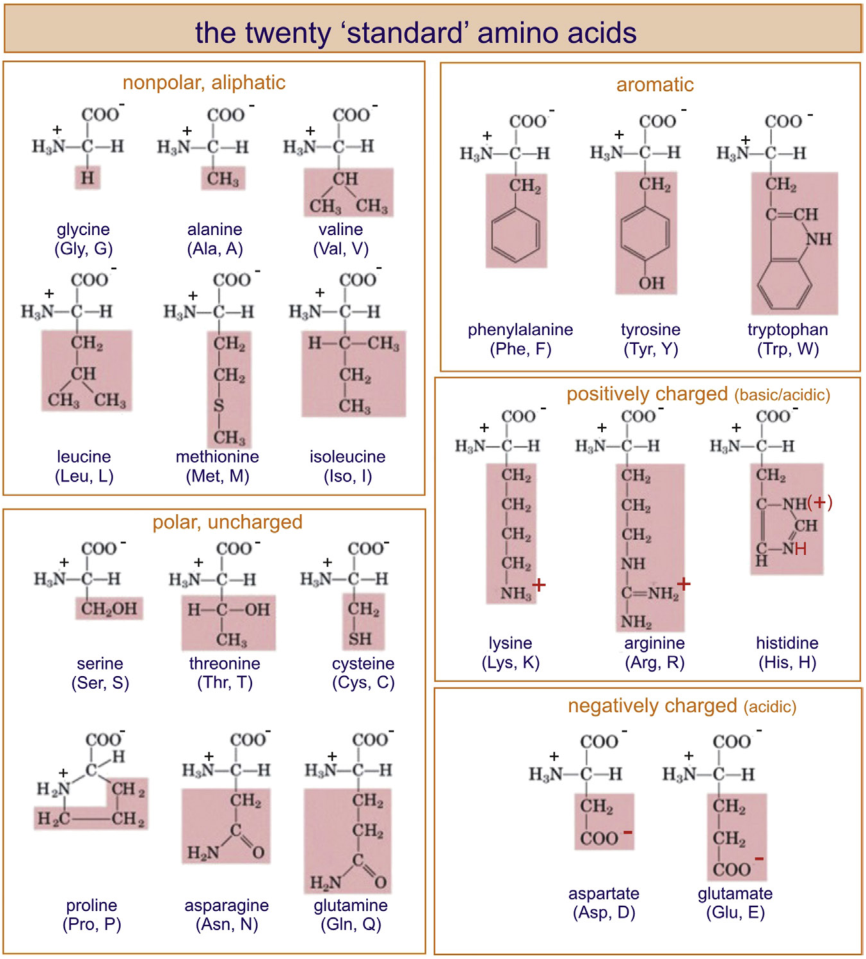


FIGURE 2-G Twenty “standard” amino acids

References

Alexander, J., Lim, D., Joughin, B.A., Hegemann, B., Hutchins, J.R., Ehrenberger, T., Ivins, F., Sessa, F., Hudecz, O., Nigg, E.A., Fry, A.M., Musacchio, A., Stukenberg, P.T., Mechtler, K., Peters, J.M., Smerdon, S.J., Yaffe, M.B., 2011. Spatial exclusivity combined with positive and negative selection of phosphorylation motifs is the basis for context-dependent mitotic signaling. Sci. Signal. 4, ra42.

Allen, K.N., Dunaway-Mariano, D., 2004. Phosphoryl group transfer: evolution of a catalytic scaffold. Trends Biochem. Sci. 29, 495–503.

Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., Mustelin, T., 2004. Protein tyrosine phosphatases review in the human genome. Cell 117, 699–711.

Amanchy, R., Periaswamy, S., Mathivanan, S., Reddy, R., Tattikota, S.G., Pandey, A., 2007. A curated compendium of phosphorylation motifs. Nat. Biotechnol. 25, 285–286.

Ames, J.B., Lim, S., Ikura, M., 2012. Molecular structure and target recognition of neuronal calcium sensor proteins. Front. Mol. Neurosci. 5, 1–12.

Anantharaman, V., Iyer, L.M., Aravind, L., 2007. Comparative genomics of protists: new insight into the evolution of eukaryotic signal transduction and gene regulation. Annu. Rev. Microbiol. 61, 453–475.

Andersson, D.C., Betzenhauser, M.J., Reiken, S., Umanskaya, A., Shiomi, T., Marks, A.R., 2012. Stress-induced increase in skeletal force requires protein kinase A phosphorylation of the ryanodine receptor. J. Physiol. 590, 6381–6387.

Archambault, J., Pan, G., Dahmus, G.K., Cartier, M., Marshall, N., Zhang, S., Dahmus, M.E., Greenblatt, J., 1998. FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase IIO. J. Biol. Chem. 273, 27593–27601.

Barbieri, C.M., Wu, T., Stock, A.M., 2013. Comprehensive analysis of OmpR phosphorylation, dimerization and DNA binding supports a canonical model for activation. J. Mol. Biol. 425, 1612–1626.

Barford, D., Flint, A.J., Tonks, N.K., 1994. Crystal structure of human protein tyrosine phosphatase 1B. Science 263, 1397–1404.

Bellinzoni, M., Wehenkel, A., Shepard, W., Alzari, P.M., 2007. Insights into the catalytic mechanism of PPM ser/thr phosphatases from the atomic resolution structures off a mycobacterial enzyme. Structure 15, 863–872.

Bode, M.A., Dong, Z., 2005. Inducible covalent posttranslational modification of histone H3. Sci. STKE. 281, re4.

Bonini, N.M., Leiserson, W.M., Benzer, S., 1993. The eyes absent gene: genetic control of cell survival and differentiation in the developing drosophila eye. Cell 72, 379–395.

Bourne, H.R., Sanders, D.A., Mc Cormick, F., 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125–132.

Boute, N., Boubekeur, S., Lacasa, D., Issad, T., 2003. Dynamics of the interaction between the insulin receptor and protein tyrosine-phosphatase 1B in living cells. EMBO Rep. 4, 313–319.

Brandt, H., Capulong, Z.L., Lee, E.Y.C., 1975. Purification and properties of rabbit liver phosphorylase phosphatase. J. Biol. Chem. 250, 8038–8044.

Brandt, D.R., Asano, T., Pedersen, S.E., Ross, E.M., 1983. Reconstitution of catecholamine-stimulated guanosinetriphosphatase activity. Biochemistry 22, 4357–4362.

Brautigan, D.L., 2013. Protein ser/thr phosphatases – the ugly ducklings of cell signalling. FEBS J. 324–345.

Bray, Denis, 2009. Wetware (A Computer in Every Living Cell). Yale University Press, New Haven & London.

Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.Y., Breitkreutz, B.J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, Z.S., Pawson, T., Gingras, A.C., Nesvizhskii, A.I., Tyers, M.A., 2010. A global protein kinase and phosphatase interaction network in yeast. Science 328, 1043–1046.

Bünemann, M., Frank, M., Lohse, M.J., 2003. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc. Natl. Acad. Sci. U.S.A. 100, 16077–16082.

Bunin, M.A., Wightman, R.M., 2000. Paracrine neurotransmission in the CNS: involvement of 5-HT. Trends Neurosci. 23, 58–59.

Burnett, G., Kennedy, E.P., 1954. The enzymatic phosphorylation of proteins. J. Biol. Chem. 211, 969–980.

Cai, M., Williams, D.C., Wang, G., Lee, B.R., Peterkofsky, A., Clore, G.M., 2003. Solution structure of the phosphoryl transfer complex between signal transducing protein 11A and the cytoplasmic domain of the glucose transporter IICB of the Escherichia coli glucose phosphotransferase system. J. Biol. Chem. 278, 25191–25206.

Casino, P., Rubio, V., Marina, A., 2010. The mechanism of signal transduction by two-component systems. Curr. Opin. Struct. Biol. 20, 763–771.

Cassel, D., Pfeuffer, D., 1987. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. Proc. Natl. Acad. Sci. U.S.A. 75, 2669–2673.

Cerione, R.A., Codina, J., Benovic, J.L., Lefkowitz, R.J., Binrbaumer, L., Caron, M.G., 1984. The mammalian beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. Biochemistry 23, 4519–4525.

Changeux, J.P., 1961. The feedback control mechanisms of biosynthetic L-threonine deaminase by L-isoleucine. Cold Spring Harb. Symp. Quant. Biol. 26, 313–318.

Charbonneau, H., Tonks, N.K., Walsh, K.A., Fischer, E.H., 1988. Human placenta protein-tyrosine phosphatase: amino acid sequence and relationship to a family of receptor-like proteins. Proc. Natl. Acad. Sci. U.S.A. 85, 7182–7186.

Cheek, S., Zhang, H., Grishin, N.V., 2002. Sequence and structure classification of kinases. J. Mol. Biol. 320, 855–881.

Cheek, S., Ginalski, K., Zhang, H., Grishin, N.V., 2005. A comprehensive update of the sequence and structure classification of kinases. BMC Struct. Biol. 5, 6.

Chen, G., Lustig, A., Weng, N.-P., 2013. T cell aging: a review of the transcriptional changes determined from genome-wide analysis. Front. Immunol. 4, 212–236.

Cherfils, J., Zeghouf, M., 2011. Chronicles of the GTPase switch. Nat. Chem. Biol. 7, 493–495.

Cherfils, J., Zeghouf, M., 2013. Regulation of small GTPases by GEFs, GAPs and GDIs. Physiol. Rev. 93, 269–309.

Chidiac, P., Hebert, T.E., Valiquette, M., Dennis, M., Bouvier, M., 1994. Inverse agonist activity of the β-adrenergic antagonists. Mol. Pharmacol. 45, 490–499.

Clapham, D.E., Neer, E.J., 1997. G-protein βγ subunits. Ann. Rev. Pharm. Tox. 37, 167–203.

Cohen, P., 2009. Keep nibbling at the edges. J. Biol. Chem. 284, 23891–23901.

Colicelli, J., 2004. Human RAS superfamily proteins and related GTPases. Sci. STKE 250, re13.

Cori, G.T., Cori, C.F., 1945. The enzymatic conversion of phosphorylase a to b. J. Biol. Chem. 158, 321–332.

Cox, A.D., Der, C.J., 2010. Ras history: the sage continues. Small GTPAses 1, 2–27.

Daaka, Y., Luttrell, L.M., Lefkowitz, R.J., 1997. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390, 88–91.

Deupi, X., Kobilka, B.K., 2010. Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. Physiol. (Bethesda) 25, 293–303.

Devedjiev, Y., Steussy, C.N., Vassylyev, D.G., 2007. Crystal structure of an asymmetric complex of pyruvate dehydrogenase kinase 3 with lipoyl domain 2 and it biological implications. J. Mol. Biol. 370, 407–416.

Dodge, K.L., Khouangsathiene, S., Kapiloff, M.S., Mouton, R., Hill, E.V., Houslay, M.D., Langeberg, L.K., Scott, J.D., 2001. mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signalling module. EMBO J. 20, 1921–1930.

Dohlman, H.G., Jones, J.C., 2012. Signal activation and inactivation by the Gα helical domain: a long-neglected partner in G protein signaling. Sci. Signal. 5 (266), re2.

Edmunds, J.W., Mahadevan, L.C., 2004. MAP kinases as structural adaoptors and enzymatic activators in transcription complexes. J. Cell Sci. 117, 3715–3723.

Egloff, M.P., Cohen, P.T., Reinemer, P., Barford, D., 1995. Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. J. Mol. Biol. 254, 942–959.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A.L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C.C., Ramachandran, C., Gresser, M.J., Tremblay, M.L., Kennedy, B.P., 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283, 1544–1548.

Endicott, J.A., Noble, M.E., Johnson, L.N., 2012. The stuctural basis for control of eukaryotic protein kinases. Ann. Rev. Biochem. 81, 587–613.

Euskirchen, G., Snyder, M., 2004. A plethora of sites. Nat. Genet. 36, 325–326.

Ferrel, J.E., 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr. Opin. Cell Biol. 14, 140–148.

Ferreon, A.C.M., Fereon, J.C., Wright, P.E., Deniz, A.A., 2013. Modulation of allostery by protein intrinsic disorder. Nature 498, 390–394.

Fischer, E.H., Krebs, E.G., 1955. Conversion of phosphorylase b to phosphorylase a in muscle extracts. J. Biol. Chem. 216, 121–132.

Fischer, E.H., Krebs, E.G., 1989. Commentory on "the phosphorylase b to a converting enzyme of rabbit skeletal muscle". Biochim. Biophys. Acta 1000, 297–301.

Fischmeister, R., Castro, L.R.V., Abi-Gerges, A., Rochais, F., Jurevicius, J., Leroy, J., Vandecasteele, G., , 2006. Compartmentation of cyclic nucleotide signalling in the heart. Cir. Res. 99, 816–828.

Fleming, I.N., Batty, I.H., Prescott, A.R., Gray, A., Kular, G.S., et al., 2004. Inositol phospholipids regulated the guanine-nucleotide exchange factor Tiam1 by facilitating its binding to the plasma membrane and regulating GDP-GTP exchange on Rac1. Biochem. J. 382, 857–865.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., Schioth, H.B., 2003. The G-proteincoupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol. Pharmacol. 63, 1256–1272.

Gallis, B., Bornstein, P., Brautigan, D.L., 1981. Proc. Natl. Acad. Sci. U.S.A. 78, 6689–6693.

Gerhart, J., Kirschner, M., 2007. The theory of facilitated variation. Proc. Natl. Acad. Sci. U.S.A. 104 (Suppl. 1), 8582–8589.

Gill, D.M., Meren, R., 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 75, 3050–3054.

Gilman, A.G., 1987. G-proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56, 615–649.

Goldberg, J., Huang, H.-B., Kwon, Y.G., Greengard, P., Nairn, A.C., Kuriyan, J., 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745–753.

Goll, M.G., Bestor, T.H., 2002. Histone modification and replacement in chromatin activation. Genes Dev. 216, 1739–1742.

Grand, S., 2003. Creation; Life and How to Make It. Harvard University Press, Cambridge, U.S.A.

Grangeasse, C., Doublet, P., Vaganay, E., Vincent, C., Deleage, G., Duclos, B., Cozzone, A.J., 1997. Characterization of a bacterial gene encoding an autophosphorylating protein tyrosine kinase. Gene 204, 259–265.

Grant, P.A., 2001. A tale of histone modifications. Genome Biol. 2 (4) Reviews0003.

Haj, F.G., Verveer, P.J., Squire, A., Neel, B.G., Bastiaens, P.I., 2002. Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. Science 295, 1708–1711.

Harvey, J.J., 1964. An unidentified virus which causes the rapid production of tumours in mice. Nature 204, 1104–1105.

Hayes, J.S., Brunton, L.L., Brown, J.H., Reese, J.B., Mayer, S.E., 1979. Hormonally specific expression of cardiac protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. 76, 1570–1574.

Hermiston, M.L., Xu, Z., Weiss, A., 2003. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. 21, 107–137.

Hofstadter, D., 1979. Godel, Escher, Bach: An Eternal Golden Braid. Basic Books, New York.

Hon, W.C., McKay, G.A., Thompson, P.R., Sweet, R.M., Yang, D.S., Wright, G.D., Berghuis, A.M., 1997. Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. Cell 89, 887–895.

Huang, F.L., Glinsmann, W.H., 1976. Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. Eur. J. Biochem. 70, 419–426.

Hubbard, M.J., Cohen, P., 1993. On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem. Sci. 18, 172–177.

Hunter, T., Sefton, B.M., 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77, 1311–1315.

Hunter, T., 2007. The age of crosstalk: phosphorylation, ubiquitinylation, and beyond. Mol. Cell 28, 730–738.

Ishibashi, T., Bottaro, D.P., Chan, A., Miki, T., Aaronson, S.A., 1992. Expression cloning of a human dual-specificity phosphatase. Proc. Natl. Acad. Sci. U.S.A. 89, 12170–12174.

Jackson, M.D., Denu, J.M., 2001. Molecular reactions of protein phosphatases–insights from structure and chemistry. Chem. Rev. 101, 2313–2340.

Janetopoulos, C., Jin, T., Devreotes, P., 2001. Receptor-mediated activation of heterotrimeric G-proteins in living cells. Science 291, 2408–2411.

Jastrzebska, B., Tsybovsky, Y., Palczewski, K., 2010. Complexes between photoactivated rhodopsin and transducin: progress and questions. Biochem. J. 428, 1–10.

Jemc, J., Rebay, I., 2007. The eyes absent family of phosphotyrosine phosphatases: properties and roles in developmental regulation of transcription. Annu. Rev. Biochem. 76, 513–538.

Johnson, L.N., Lewis, R.J., 2001. Structural basis for control by phosphorylation. Chem. Rev. 101, 2209–2242.

Johnston, C.A., Siderovski, D.P., 2007. Receptor-mediated activation of heterotrimeric G-proteins: current structural insights. Mol. Pharmacol. 72, 219–230.

Jung, K., Luitpold, F., Behr, S., Heermann, R., 2012. Histidine kinases and response regulators in networks. Curr. Opin. Microbiol. 15, 118–124.

Jura, N., Endres, N.F., Engel, K., Deindl, S., Das, R., Lamers, M.H., Wemmer, D.E., Zhang, X., Kuriyan, J., 2009. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. Cell 137, 1293–1307.

Jura, N., Zhang, X., Endres, N.F., Seeliger, M.A., Schindler, T., Kuriyan, J., 2011. Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. Mol. Cell 42, 9–22.

Jura, N., Zhang, X., Endres, N.F., Seelinger, M.A., Schindler, T., Kuriyan, J., 2012. Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. Mol. Cell 42, 9–22.

Kemp, B.E., Graves, D.J., Benjamini, E., Krebs, E.G., 1977. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J. Biol. Chem. 252, 4888–4894.

Kenakin, T., Miller, L.J., 2010. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity of new drug discovery. Pharmacol. Rev. 62, 265–304.

Kholodenko, B.N., 2000. Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. Eur. J. Biochem. 267, 1583–1588.

Kim, D.J., Forst, S., 2001. Genomic analysis of the histidine kinase family in bacteria and archaea. Microbiol 147, 1197–1212.

Kirsten, W.H., Carter, R.E., Pierce, M.I., 1962. studies on the relationship of viral infections to leukemia in mice: the accelerating agent in AKR mice. Cancer 15, 750–758.

Krebs, E.G., Fischer, E.H., 1956. The phosphorylase b to a converting enzyme of rabbit skeletal muscle. Biochim. Biophys. Acta 20, 150–157.

Kundig, W., Ghosk, S., Roseman, S., 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphor-transferase system. Proc. Natl. Acad. Sci. U.S.A. 52, 1067–1074.

Landry, C.R., Levy, E.D., Michnick, S.W., 2009. Weak functional constraints on phosphoproteomes. Trends Genet. 25, 193–197.

Lipmann, F.A., Levene, P.A., 1932. Serine phosphoric acid obtained on hydrolysis of vitellinic acid. J. Biol. Chem. 98, 107–112.

Lee, K.K., Workman, J.L., 2007. Histone acetyltransferase complexes: one size doesn't fit all. Nat. Rev. Mol. Biol. 8, 284–295.

Lehoucqu, R., 10 November 2012. Le Monde Sci. Technol.

Levene, P.E., Alsberg, C.L., 1906. The cleavage products of vitellin. J. Biol. Chem. 2, 127–133.

Levy, E.D., Landry, C.R., Michnick, S.W., 2009. How perfect can protein interactomes be? Sci. Signal 2 (60), pe11.

Li, X., Oghi, K.A., Zhang, J., Krones, A., Bush, K.T., Glass, C.K., Nigam, S.K., Aggarwal, A.K., Maas, R., Rose, D.W., Rosenfeld, M.G., 2003. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426, 247–254.

Lim, J., Hotchin, N.A., 2012. Signalling mechanisms of the leukocyte integrin αMβ2: current and future perspectives. Biol. Cell 104, 631–640.

Lima, C.D., 2005. Inducing interactions with CTD. Nat. Struct. Mol. Biol. 12, 102–103.

Liokatis, S., Stützer, A., Elsässer, S.J., Theillet, F.X., Klingberg, R., van Rossum, B., Schwarzer, D., Allis, C.D., Fischle, W., Selenko, P., 2012. Phosphorylation of histone H3 Ser10 establishes a hierarchy for subsequent intramolecular modification events. Nat. Struct. Mol. Biol. 19, 819–823.

Lipmann, F., Levene, P.E., Rimington, C., 1926. Some phosphorus compounds of milk. Biochem. J. 20, 777–790.

Liu, W., Northup, J.K., 1998. The helical domain of a G protein α subunit is a regulator of its effector. Proc. Natl. Acad. Sci. U.S.A. 95, 12878–12883.

Logothis, D.E., Kurachi, Y., Galper, J., Neer, E.J., Clapham, D.E., 1987. Purified subunits of GTP-binding proteins regulate muscarinic K+ channel activity in the heart. Nature 325, 321–326.

Lowe, D.G., Chang, M.S., Hellmiss, R., Chen, E., Singh, S., Garbers, D.L., Goeddel, D.V., 1989. Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. EMBO J. 8, 1377–1384.

Lowery, D.M., Lim, D., Yaffe, M.B., 2005. Structure and function of polo-like kinases. Oncogene 24, 248–259.

Lu, C., Mi, L.Z., Grey, M.J., Zhu, J., Graef, E., Yokoyama, S., Springer, T.A., 2010. Structural evidence for loose linkage between ligand binding and kinase activation in the epidermal growth factor receptor. Mol. Cell Biol. 30, 5432–5443.

Macek, B., Mijakovic, I., 2011. Site-specific analysis of bacterial phosphoproteomes. Proteomics 11, 3002–3011.

Mahadevan, L.C., Willis, A.C., Barratt, M.J., 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. Cell 65, 775–783.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. Science 298, 1912–1934.

Manning, G., Young, S.L., Miller, W.T., Zha, Y., 2008. The protist, monosiga brevicollis, has tyrosine kinase signaling network more elaborate and diverse than found in known metazoan. Proc. Natl. Acad. Sci. U.S.A. 105, 9674–9679.

Markby, D.W., Onrust, R., Bourne, H.R., 1993. Separate GTP binding and GTPase activating domains of a G alpha subunit. Science 262, 1895–1901.

Maynes, J.T., Bateman, K.S., Cherney, M.M., et al., 2001. Crystal structure of the tumor-promotor okadaic acid bound to protein phosphatase-1. J. Biol. Chem. 276, 44078–44082.

McBride, A., Hardie, D.G., 2009. AMP-activated protein kinase–a sensor of glycogen as wells as AMP and ATP? Acta Physiol. 196, 99–113.

Michell, R.H., 1975. Inositol phospholipids in cell surface receptor function. Biochim. Biophys. Acta 415, 81–147.

Mildvan, A.S., 1997. Mechanisms of signalling and related enzymes. Proteins 29, 401–416.

Miller, M.L., Jensen, L.J., Diella, F., Jorgensen, C., Tinti, M., Li, L., Hsiung, M., Parker, S.A., Bordeaux, J., Sicheritz-Ponten, T., Olhovsky, M., Pasculescu, A., Alexander, J., Knapp, S., Blom, N., Bork, P., Li, S., Cesareni, G., Pawson, T., Turk, B.E., Yaffe, M.B., Brunak, S., Linding, R., 2008. Linear motif atlas for phosphorylation-dependent signaling. Sci. Signal. 1, ra2.

Minassian, B.A., Lee, J.R., Herbrick, J.A., Huizenga, J., Soder, S., et al., 1998. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nat. Genet. 20, 171–174.

Mohi, M.G., Neel, B.G., 2007. The role of Shp2 (PTPN11) in cancer. Curr. Opin. Gen. Dev. 17, 23–30.

Mok, J., Kim, P.M., Lam, H.Y.K., Piccirillo, S., Zhou, X., Jeschke, G.R., Sheridan, D.L., Parker, S.A., Desai, V., Jwa, M., Cameroni, E., Niu, H., Good, M., Remenyi, A., Nianhan Ma, J.L., Sheu, Y.J., Sassi, H.E., Sopko, R., Chan, C.S.M., De Virgilio, C., Hollingsworth, N.M., Kim, W.A., Stern, D.F., STillman, B., Andrews, B.J., Gerstein, M.B., Snyder, M., Turk, B.E., 2010. Deciphering protein kinase specificity through large-scale analysis of yeast phosphorylation site motifs. Sci. Signal. 3 (109), ra12.

Monetti, M., Nagaraj, N., Sharma, K., Mann, M., 2011. Large-scale phosphosite quantification in tissues by a spike-in SILAC method. Nat. Methods 8, 655–658.

Monod, J., Changeux, J.P., Jacob, F., 1963. Allosteric proteins and cellular control systems. J. Mol. Biol. 6, 306–329.

Monsey, J., Shen, W., Schlesinger, P., Bose, R., 2010. Her4 and Her2/neu tyrosine kinase domains dimerized and activate in a reconstituted in vitro system. J. Biol. Chem. 285, 7035–7044.

Moorhead, G.B.G., de Wever, V., Templeton, G., Kerk, D., 2009. Evolution of protein phosphatases in plants and animals. Biochem. J. 417, 401–409.

Morris, A.J., Malbon, C.C., 1999. Physiological regulation of G protein-linked signaling. Physiol. Rev. 79, 1373–1430.

Munro, S., Cuthbertson, D.J., Cunningham, J., Sales, M., Cohen, P.T., 2002. Human skeletal muscle expresses a glycogen-targeting subunit of PP1 that is identical to the insulin-sensitive glycogen-targeting subunit G(L) of liver. Diabetes 5, 591–598.

Munro, S., Ceulemans, H., Bollen, M., Diplexcito, J., Cohen, P.T., 2005. A novel glycogen-targeting subunit of protein phosphatase 1 that is regulated by insulin and shows differential tissue distribution in humans and rodents. FEBS J. 272, 1478–1489.

Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., Kuriyan, J., 2003. Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell 112, 859–871.

Nagar, B., Hantschel, O., Seeliger, M., Davies, J.M., Weis, W.I., Superti-Furga, G., Kuriyan, J., 2006. Organization of the SH3-SH2 unit in active and inactive forms of the c-Abl tyrosine kinase. Mol. Cell 21, 787–798.

Neal, S.E., Eccleston, J.F., Hall, A., Webb, R., 1988. Kinetic analysis of the hydrolsysis of GTP by p21N-ras. The basal GTPase mechanism. J. Biol. Chem. 263, 19718–19722.

Nitschke, F., Wang, P., Schmieder, P., Girard, J.M., Awrey, D.E., et al., 2013. Hyperphosphorylation of glucosyl C6 carbons and altered structure of glycogen in the neurodegenerative epilepsy Lafora disease. Cell Metab. 17, 756–767.

Nobles, K.N., Xiao, K., Ahn, S., Shukla, A.K., Lam, C.M., Rajagopal, S., Strachan, R.T., Huang, T.-Y., Bressler, E.A., Hara, M.R., Shenoy, S.K., Gygi, S.P., Lefkowitz, R.J., 2011. Distinct phosphorylation sites on the β2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. Sci. Signal. 4, ra51.

Obenauer, J.C., Cantley, L.C., Yaffe, M.B., 2003. Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 31, 3635–3641.

Offermanns, S., 2003. G-proteins as transducers in transmembrane signalling. Prog. Biophys. Mol. Biol. 83, 101–130.

Oldham, W., Hamm, H.E., 2008. Heterotrimeric G protein activation by G-protein-coupled receptors. Nat. Rev. Mol. Biol. 9, 60–71.

Olsen, J.V., Blagoev, F., Macek, B., et al., 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648.

Pérez, J., Castañeda-García, A., Jenke-Kodama, H., Müller, R., 2008. Eukaryotic-like protein kinases in the prokaryotes and the myxobacterial kinome. Proc. Natl. Acad. Sci. U.S.A. 105, 15950–15955.

Perlmann, G.E., 1955. The nature of phosphorus linkages in phosphoproteins. Adv. Protein Chem. 10, 1–30.

Pigliucci, M., Boudry, M., 2011. Why Machine-information metaphors are bad for science and science education. Sci. Educ. 20, 453–471.

Printen, J.A., Brady, M.J., Saltiel, A.R., 1997. PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. Science 275, 1475–1478.

Pylayeva-Gupta, Y., Graocka, E., Bar-Sagi, D., 2011. Ras oncogenes: weaving a tumorigenic web. Nat. Rev. Cancer 11, 761–774.

Rall, T.W., Sutherland, E.W., Berthet, J., 1957. The relationship of epinephrine and glucagon to liver phosphorylase: effect of epinephrine and glycogen on the reactivation of phosphorylase in liver homogenates. J. Biol. Chem. 224, 463–475.

Rasmussen, S.G.F., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.A., et al., 2011. Crystal structure of the β2 adrenergic receptor Gs complex. Nature 477, 549–557.

Rauen, K.A., 2013. The RASopathies. Ann. Rev. Genomics Hum. Genet. 14, 17.1–17.15.

Red Brewer, M., Choi, S.H., Alvarado, D., Moravcevic, K., Pozzi, A., et al., 2009. The juxtamembrane region of the EGF receptor functions as an activation domain. Mol. Cell 34, 641–651.

Reményi, A., Good, M.C., Lim, W.A., 2006. Docking interactions in protein kinase and phosphatase networks. Curr. Opin. Struct. Biol. 16, 676–685.

Reuveny, E., Slesinger, P.A., Inglese, J., 1994. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. Nature 370, 143–146.

Roach, P.J., Depaoli-Roach, A.A., Hurley, T.D., Tagliabracci, V.S., 2012. Glycogen and its metabolism: some new developments and old themes. Biochem. J. 441, 763–787.

Rodbell, M., Birnbaumer, L., Pohl, S.M., Krans, H.M., 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver: an obligatory role of guanylnucleotides in glucagon action. J. Biol. Chem. 246, 1877–1882.

Rosenbaum, D., Zhang, C., Lyons, J.A., et al., 2011. Structure and function of an irreversible agonist-β2 adrenoreceptor complex. Nature 469, 236–240.

Rossetto, D., Avvakumov, N., Côte, J., 2012. Histone phosphorylation: a chromatin modification involved in diverse nuclear events. Epigenetics 7, 1098–1108.

Russell, P., Nurse, P., 1986. Cdc25þ functions as an inducer in the mitotic control of fission yeast. Cell 45, 145–153.

Salmeen, A., Andersen, J.N., Myers, M.P., Tonks, N.K., Barford, D., 2000. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. Mol. Cell 6, 1401–1412.

Scheeff, E.D., Bourne, P.E., 2005. Structural evolution of the protein kinase-like superfamily. PLoS Comput. Biol. 1, e49.

Scheffzek, K., Ahmadian, M.R., Wittinghofer, A., 1999. GTPase-activating proteins: helping hands to complement the active site. Trends Biochem. Sci. 257–262.

Segal, A.W., 2005. How neutrophils kill microbes. Annu. Rev. Immunol. 23, 197–223.

Shenoy, S.K., Drake, M.T., Nelson, C.D., Houtz, D.A., Xiao, K., Madabushi, S., Reiter, E., Premont, R.T., Lichtarge, O., Lefkowitz, R.J., 2006. β-arrestindependent, G protein-independent ERK1/2 activation by the β2 adrenergic receptor. J. Biol. Chem. 281, 1261–1273.

Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., et al., 1983. Three human transforming genes are related to the viral ras oncogenes. Proc. Natl. Acad. Sci. U.S.A. 80, 2112–2116.

Shimonaka, M., Katagiri, K., Nakayama, T., Fujita, N., Tsuruo, T., et al., 2003. Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. J. Cell Biol. 161, 417–427.

Shin, S.Y., Rath, O., Choo, S.M., Fee, F., McFerran, B., Kolch, W., Cho, K.H., 2009. Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway. J. Cell Sci. 122, 425–435.

Siderovski, D.P., Willard, F.S., 2005. The GAPs, GEFs and GDIs of heterotrimeric G-protein alpha subunits. Int. J. Biol. Sci. 1, 51–61.

Smart, J.E., Oppermann, H., Czernilofsky, A.P., Pu, A.F., Erikson, R.L., Bishop, J.M., 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src). Proc. Natl. Acad. Sci. U.S.A. 78, 6013–6017.

Smienk, H.G.F., Calvo, D., Razquin, P., Domínguez, E., Mata, L., 2012. Single laboratory validation of a ready-to-use phosphatase inhibition assay for detection of okadaic acid toxins. Toxins 4, 339–352.

Smith, C.A., Toth, M., Frase, H., Byrnes, L.J., Vakulenko, S.B., 2012. Aminoglycoside 2′-phosphotransferase IIIa (APH(2′)-IIIa) prefers GTP over ATP: structural templates for nucleotide recognition in the bacterial aminoglycoside-2′ kinases. J. Biol. Chem. 287, 12893–12903.

Sprang, S.R., Acharya, K.R., Goldsmith, E.J., Stuart, D.I., Varvill, K., Fletterick, R.J., Madsen, N.B., Johnson, L.N., 1988. Structural changes in glycogen phosphorylase induced by phosphorylation. Nature 336, 215–221.

Stephenson, R.P., 1956. A modification of receptor theory. Br. J. Pharmacol. 11, 379–393.

Suga, H., Dacre, M., de Mendoza, A., Shalchian-Tabrizi, K., Manning, G., Ruiz-Trillo, I., 2012. Genomic survey of pre-metazoans show deep conservation of cytoplasmic tyrosine kinases and multiple radiations of receptor tyrosine kinases. Sci. Signal. 5, ra35.

Sutherland, E.W., Rall, T.W., Menon, T., 1962. Adenyl cyclase: distribution, preparation and properties. J. Biol. Chem. 237, 1220–1227.

Tall, G.G., 2013. Ric-8 regulation of heterotrimeric G-proteins. J. Recept Signal Transduct. Res. 33, 139–143.

Tan, M., Luo, H., Lee, S., Jin, F., Yang, J.S., Montellier, E., Buchou, T., Cheng, Z., Rousseaux, S., Rajagopal, N., Lu, Z., Ye, Z., Zhu, Q., Wysocka, J., Ye, Y., Khochbin, S., Ren, B., Zhao, Y., 2011. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146, 1016–1028.

Taylor, S.S., Ilouz, R., Zhang, P., Kornev, A.P., 2012a. Assembly of allosteric macromolecular switches: lessons from PKA. Nat. Rev. Mol. Cell Biol. 13, 646–658.

Taylor, S.S., Keshwani, M.M., Steichen, J.M., Kornev, A.P., 2012b. Evolution of the eukaryotic protein kinases as dynamic molecular switches. Phil. Trans. R. Soc. 367, 2517–2528.

Ten Eyk, L.F., Taylor, S.S., Kornev, A.P., 2008. Conserved spatial patterns across the protein kinase family. Biochim. Biophys. Acta 1784, 238–243.

Terrak, M., Kerff, F., Langsetmo, K., Tao, T., Dominiquez, R., 2004. Structural basis of protein phosphatases 1 regulation. Nature 429, 780–784.

Tesmer, J.J., Berman, D.M., Gilman, A.G., Sprang, S.R., 1997. Structure of RGS4 bound to AlF4—activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell 89, 251–261.

Thaller, M.C., Schippa, S., Rossolini, G.M., 1998. Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Protein Sci. 7, 1647–1652.

Tiganis, T., Bennett, A.M., 2007. Protein tyrosine phosphatase function: the substrate perspective. Biochem. J. 402, 1–15.

Tiganis, T., 2013. PTP1B and TCPTP – nonredundant phosphatases in insulin signaling and glucose homeostasis. FEBS J. 280, 445–458.

Tonks, N.K., Diltz, C.D., Fischer, E.H., 1988. Purification of the majore protein-tyrosine phosphatases of human placenta. J. Biol. Chem. 263, 6722–6730.

Toyoshima, C., 2009. How Ca^{2+} ATPase pumps ions across the sarcoplasmic reticulum membrane. Biochim. Biophys. Acta 1793, 941–946.

Tremblay, M.L., 2009. The PTP family photo album. Cell 136, 213–214.

Tsui, H.W., Siminovitch, K.A., de Souza, L., Tsui, F.W., 1993. Motheaten and viable motheaten mice have mutations in the hematopoietic cell phosphatase gene. Nat. Genet. 4, 124–129.

Uversky, V.N., Oldfield, C.K., Dunker, A.K., 2008. Intrinsically disordered proteins in human diseases: introducing the D^2 concept. Annu. Rev. Biophys. 37, 215–246.

Varon, C., Tatin, F., Moreau, V., Van Obberghen-Schilling, E., Fernandez-Sauze, S., Reuzeau, E., Kramer, I., Génot, E., 2006. Transforming growth factor beta induces rosettes of podosomes in primary aortic endothelial cells. Mol. Cell Biol. 26, 3582–3594.

Vincent, C., Doublet, P., Grangeasse, C., Vaganay, E., Cozzone, A.J., Duclos, B., 1999. Cells of *Escherichia coli* contain a protein-tyrosine kinase, Wzc, and a phosphotyrosine-protein phosphatase. Wzb. J. Bacteriol. 181, 3472–3477.

Waddington, C.H., 1942. The epigenotype. Endeavor 1, 18–20.

Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W., Bollen, M., 2003. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. J. Biol. Chem. 278, 18817–18823.

Waldo, G.L., Ricks, T.K., Hicks, S.N., Cheever, M.L., Kawano, T., Tsuboi, K., Wang, X., Montell, C., Kozasa, T., Sondek, J., Harden, T.K., 2010. Kinetic scaffolding mediated by a phospholipase C-beta and Gq signaling complex. Science 330, 974–980.

Walsh, D.A., Perkins, J.P., Krebs, E.G., 1968. An adenosine 3′5′-monophosphate-dependant protein kinase from rabbit skeletal muscle. J. Biol. Chem. 243, 3763–3765.

Wang, Z., Shen, D., Parsons, D.W., Bardelli, A., Sager, J., et al., 2004. Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science 304, 1164–1166.

Welch, E.J., Jones, B.W., Scott, J.D., 2010. Networking with AKAPs: context-dependent regulation of anchored enzymes. Mol. Interv. 10, 86–89.

Wiedemann, A., Patel, J.C., Lim, J., Tsun, A., van Kooyk, Y., Caron, E., 2006. Two distinct cytoplasmic regions of the beta2 integrin chain regulate RhoA function during phagocytosis. J. Cell Biol. 172, 1069–1079.

Yang, X.-J., 2005. Multisite protein modification and intramolecular signaling. Oncogene 24, 1653–1662.

Yoshida, T., Qin, L., Egger, L.A., Inouye, M., 2006. Transcription regulation of ompF and ompC by a single transcription factor, OmpR. J. Biol. Chem. 281, 17114–17123.

Yun, M., Wu, J., Workman, J.L., Bing, L., 2011. Readers of histone modifications. Cell Res. 21, 564–578.

Zhang, X., Gureasko, J., Shen, K., Cole, P.A., Kuriyan, J., 2006. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125, 1137–1149.

Zhang, L., Malik, S., Kelley, G.G., Kapiloff, M.S., Smrcka, A.V., 2011. Phospholipase C epsilon scaffolds to muscle-specific A kinase anchoring protein (mAKAPbeta) and integrates multiple hypertrophic stimuli in cardiac myocytes. J. Biol. Chem. 286, 23012–23021.

Zozulya, S., Stryer, L., 1992. Calcium-myristoyl protein switch. Proc. Natl. Acad. Sci. U.S.A. 89, 11569–11573.

CHAPTER

3

Regulation of Muscle Contraction by Adrenoceptors

But when the blast of war blows in our ears,
Then imitate the action of the tiger;
Stiffen the sinews, summon up the blood,
Disguise fair nature with hard-favour'd rage;
Then lend the eye a terrible aspect…

William Shakespeare, Henry V, II. ii. 1

We begin with a description of the receptors for the catecholamines, adrenaline, noradrenaline, and dopamine. These provide the basic paradigms by which the main classes of hormone, neurotransmitter, and drug receptors have been defined. We then place these receptors in the context of the regulation of muscle contraction, cardiac and vascular smooth muscle, and will elaborate on their signaling mechanism, with particular emphasis on two classic G-protein effectors, adenylyl cyclase and phospholipase C (PLC). We will show how the intervention of G-protein-coupled receptor kinases and arrestins not only arrest signaling through these two classic effectors but also bring about a large set of new signaling events that play an essential, long-term role in the functioning of cardiomyocytes.

CATECHOLAMINES

Adrenaline

Adrenaline (epinephrine) belongs to the group of hormones and neurotransmitters known as catecholamines. The other members are noradrenaline (norepinephrine) and dopamine. Its structures and synthesis pathway are shown in Figure 3-1. Adrenaline is secreted by chromaffin cells of the adrenal medulla. These cells are under the control of acetylcholine secreted from nerves of the sympathetic nervous system

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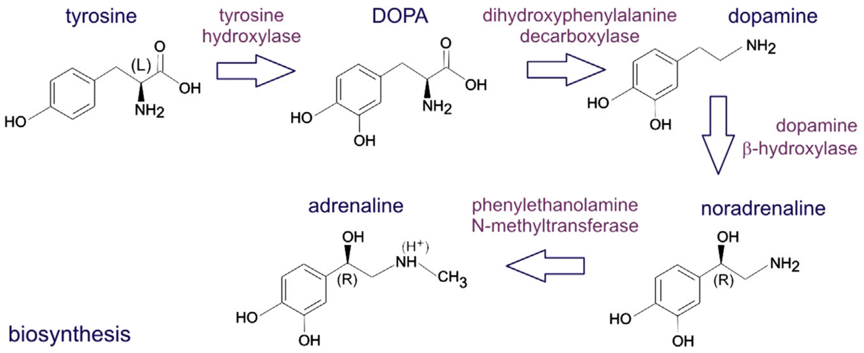


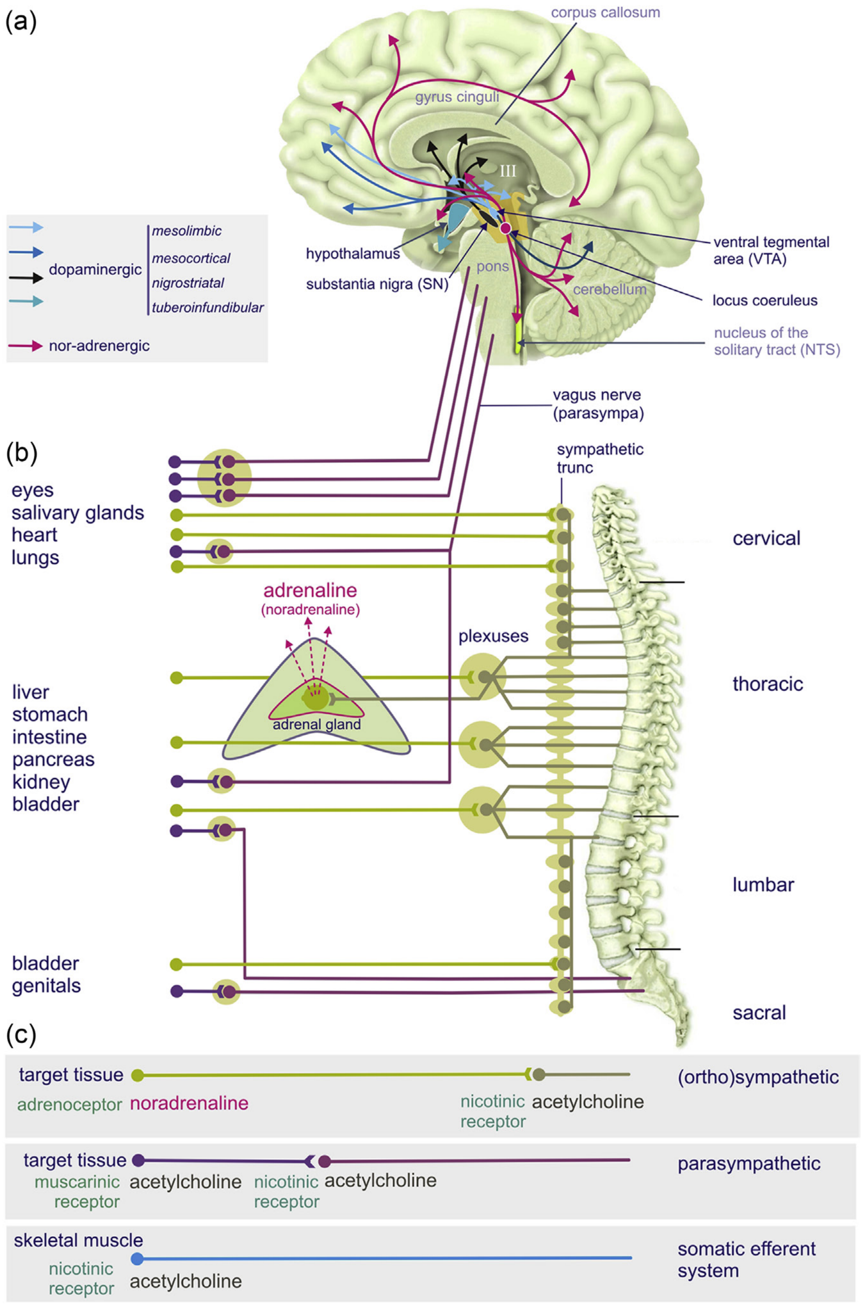
FIGURE 3-1 Biosynthesis of catecholamines.

(Figure 3-2). Adrenaline is released in anticipation of danger, preparing the body for fight or flight (Cannon, 1929).

> As Cannon has pointed out, this secretion (the internal secretion of the medulla of the suprarenal bodies) is poured into the blood during conditions of stress, anger, or fear, and acts as a potent reinforcement to the energies of the body. It increases the tone of the blood vessels as well as the power of the heart's contraction, while it mobilizes the sugar bound up in the liver, so that the muscles may be supplied with the most readily available source of energy in the struggle to which these emotional states are the essential precursors or concomitants. ***Starling (1923)***

The immediate actions are those of arousal: the sweat glands sweat, body-hair stands on end, the pupils dilate to gather more light, the bronchi dilate to improve oxygen supply, and the heart quickens and increases its force of

FIGURE 3-2 Catecholamine-producing neurons in the central- and autonomic-peripheral nervous system. (a) The midbrain has a number of prominent anatomical sites (called nuclei or loci) that produce dopamine and noradrenaline. For dopamine the substantia nigra, the ventral tegmental area, and the hypothalamus are highlighted. Projections (axons) go a long way into the brain, some of which are involved in the regulation of reward-motivated behavior (mesolimbic, mesocortical, nigrostriatal, tuberoinfundibular circuits). Dopamine neurons also project into the cerebellum. The locus coeruleus is generally considered the major site of noradrenaline production and its axons too reach many parts of the brain. (b) The autonomic peripheral nervous system is separated into the (ortho)sympathetic and the parasympathetic. The sympathetic nerves all sprout from the spine. There axons form synapses at different levels: certain terminate in the sympathetic trunk, others in big neuronal plexuses (celiac and mesenteric) and one branch of nerves terminates in the medulla of the adrenal gland. They all produce acetylcholine which regulates opening of nicotinic receptors at the downstream neuron. These, in turn, carry the signal to the target tissues and release noradrenaline. The adrenal gland forms an exception, its neurons have converted into chromaffin cells and these mainly produce adrenaline and only a little noradrenaline. The parasympathetic nerves originate in the pons and medulla area of the brain or they sprout from the sacral part of the spinal column. There axons form synapses near the target tissue. They release acetylcholine and the message is transmitted via nicotinic receptors. The postsynaptic neurons, which enervate the target tissues, also produce acetylcholine but the signal is transduced by muscarinic receptors. (c) Schematic representation of the neuronal connections of the (ortho)sympathetic and parasympathetic nervous system.


(a)
corpus callosum
gyrus cinguli
III
mesolimbic
mesocortical
nigrostriatal
tuberoinfundibular
dopaminergic
nor-adrenergic
hypothalamus
substantia nigra (SN)
pons
cerebellum
ventral tegmental area (VTA)
locus coeruleus
nucleus of the solitary tract (NTS)
vagus nerve (parasympa)
(b)
sympathetic trunc
eyes
salivary glands
heart
lungs
cervical
adrenaline
(noradrenaline)
plexuses
adrenal gland
liver
stomach
intestine
pancreas
kidney
bladder
thoracic
lumbar
bladder
genitals
sacral
(c)
target tissue
adrenoceptor
noradrenaline
nicotinic receptor
acetylcholine
(ortho)sympathetic
target tissue
muscarinic receptor
acetylcholine
nicotinic receptor
acetylcholine
parasympathetic
skeletal muscle
nicotinic receptor
acetylcholine
somatic efferent system

contraction. Then, through dilation of relevant vessels and constriction of others, the blood supply to the myocardium and selected skeletal muscles is increased, while the supply to the skin and visceral organs is reduced, postponing digestive and anabolic processes. Metabolic fuel stores are also mobilized (initially breakdown of glycogen in the liver and muscles, and ultimately the catabolism of fat). Thus the secretion of adrenaline is familiar in the context of emergencies. However, it important to remember that the adrenal gland continuously produces low levels of the hormone and that adrenaline is also released into the circulation during exercise.

Noradrenaline

Noradrenaline differs from adrenaline by the presence of an *N*-methyl group on the side chain (Figure 3-1). In humans, noradrenaline is also secreted by chromaffin cells of the adrenal medulla (but much less than adrenaline), but its main action occurs through release from peripheral sympathetic neurons at the level of effector organs and by neurons of the central nervous system (Figure 3-2). With respect to its peripheral effects both from neurons and adrenal medulla, they resemble the action of adrenaline (two systems working in parallel). Sympathetic peripheral neurons that liberate noradrenaline also liberate ATP and certain neuropeptides (e.g., neuropeptide Y). The brain production of noradrenaline is mainly located in neurons that originate in the pons and medulla oblongata (brain stem). The locus coeruleus, situated in the pons, is regarded as the principal site for its synthesis. Neurons in this locus continuously release noradrenaline during wakefulness, they decrease their activity during slow-wave sleep and they are silent during rapid eye movement (RED) sleep. It is generally assumed that the central nervous actions of noradrenaline lead to a state of arousal that is essential for orientation to novel stimuli, selective attention, and vigilance (all important aspects of learning). Noradrenaline may also play a role in reexperiencing strong aversive events through intrusive memories and nightmares (Usher et al., 1999; Southwick et al., 1999).

NOMENCLATURE

Adrenaline (A) owns its name because of its production in the adrenal gland. Because of patent matters the Americans were refrained from this term and have adopted epinephrine. Adrenaline derives from the Latin root (ad ren(es)) and epinephrine from the Greek (epi nephr(os). The origin of the prefix "nor," as in noradrenaline (NA), seems to refer to normal; the demethylated form of the parent compound. Source: http://en.wikipedia.org/wiki/Nor-. Some references mention that it refers to the German "nitrogen ohne radikal," for the reason that the methyl group acts as a "radical"; here defined as a group ready for chemical reaction.

NOMENCLATURE *(cont'd)*

We use the conventional British nomenclature, adrenaline and noradrenaline, although the recommended international nonproprietary names (rINNs) are now epinephrine and norepinephrine (also known as American nomenclature). Source www.who.int/medicines/services/inn/.

THE AUTONOMIC NERVOUS SYSTEM

The autonomic nervous system (or autonomous, involuntary) covers the innervation of organs and viscera. Anatomically it appears as part of the peripheral nervous system, which includes the voluntary somatic component innervating skeletal muscles (both efferent motor and afferent sensory nerves).

Globally, it controls processes, such as the contraction and relaxation of smooth muscle, the exocrine secretions and some endocrine secretion (pancreas, adrenal gland), heart rate, the carbohydrate and lipid metabolism (especially in the liver and muscles). The system is subdivided into three components: (1) the (ortho) sympathetic, (2) the parasympathetic, and (3) gastrointestinal or enteric system.

Add 1: The cell bodies of preganglionic sympathetic neurons constitute the lateral horn of the gray matter of the thoracic and lumbar spinal cord. Their axons leave the spinal cord in between the vertebral discs and they synapse at the level of the paravertebral chain ganglia (sympathetic trunk or "great sympathetic nerve") or in prevertebral ganglia located further downstream (celiac and mesenteric ganglia). The postganglionic neurons contact the effector organs. The adrenal gland forms an exception, the postganglionic neurons of the medulla are the adrenaline-producing chromaffin cells (the medulla is a modified sympathetic ganglion).

Add 2: The cell bodies of preganglionic parasympathetic neurons constitute the nuclei of cranial nerves in the brain stem and in the sacral spinal cord. The axons from the brain stem give rise to the cranial nerves (e.g., the oculomotor nerve (eye), facial nerve, the glossopharyngeal nerve (salivary glands and nasopharynx area), or the vagus nerve (innervating thoracic and abdominal organs (viscera))). Axons from the sacral spinal cord give rise to pelvic nerves (erector nerve for instance) and these contact postganglionic neurons located on or in the wall of the target organs (bladder, genitals).

continued

THE AUTONOMIC NERVOUS SYSTEM *(cont'd)*

Add 3: The gastrointestinal, or enteric, system forms a network apart and is generally considered to be our second brain. It receives signals from both the sympathetic and parasympathetic neurons (splanchnic and vagal + sacral nerves respectively), which enter through the mesentery. It controls peristalsis, waves of contraction of smooth muscle, and secretion of digestive juices. It comprises a network of plexuses: the myenteric or Auerbach's plexuses which reside in between the two muscle layers, Meissner's plexuses, which are found in the submucosal layer and Issawa's plexuses which occur in the lamina propria. Figure 3-2 illustrates the neurotransmitters that are operational in the autonomic nervous system.

As a rule of the thumb, (ortho)sympathetic and parasympathetic actions are functionally antagonistic; one causes contraction, the other relaxation, one increases the rhythm, the other reduces (heartbeat, neural action potentials), one increases secretion, the other prevents it. To our knowledge, all organs are innervated by both systems but not necessarily in equal proportions.

Dopamine

Dopamine-producing neurons are found in four anatomical regions of the midbrain, the ventral tegmental area (VTA), the substantia nigra (SN), the hypothalamus, and the zona incerta. The first three take part in four different reward/motor-control circuits (Figure 3-2). The mesolimbic circuit: comprising dopamine-containing neurons that originate in the VTA (in midbrain) and innervate the nucleus accumbens (NAc) (ventral striatum), amygdala, hippocampus, and the prefrontal cortex. This circuit is known for its role in memory and in motivation. An excess of activity may lead to schizophrenia and hallucinations. The mesocortical circuit, which also originates in the VTA but innervates only the prefrontal and orbitofrontal cortex. This circuit is also involved in schizophrenia and hallucinations. The nigrostriatal circuit comprises dopamine-containing neurons that originate from the SN (located just ventrolateral of the VTA) and innervate the caudate nucleus and the putamen (which together form the "dorsal striatum"). They have a motor control function. This circuit is affected in Parkinson's disease, a pathology characterized by the loss of dopamine-containing neurons and resulting in a number of motor abnormalities such as tremor at rest, postural instability, slowness of movement and rigidity. It now appears that degeneration of nerve cells is not limited to the SN, it is also observed in several other regions of the central and peripheral nervous systems. For instance, deep brain stimulation of the zona incerta (subthalamus region), another area with dopamine-producing neurons, has shown promising effects in treating Parkinson's disease. The tuberoinfundibular circuit originates in the hypothalamus and innervates the pituitary. It is known for its control of secretion into the blood of, among others, prolactin and ACTH (corticotrophin). Both play a role in determining our behavior: seeking pleasure and/or avoiding pain (fight or flight). Dopamine binds G-protein-coupled receptors (GPCR), the DRD family, comprising five members (DRD1–5). We will not return to dopamine in this chapter.

DOPAMINE AND REWARD

The dopamine reward hypothesis posits that dopamine promotes reward-related activities. It promotes "wanting" hence animals will work harder to obtain food rewards when dopamine signaling increases (bursts of neurotransmitter release). Dopamine is also postulated to promote learning associations between food rewards and the environment where they are found (it helps you to remember the location of the bakery shop). Certain street drugs (for instance, cannabis, cocaine, methamphetamine, or heroin) promote dopamine release (Palmitter, 2007; Berridge, 2006).

Web sources

- Animation of mode of action of street drugs http://learn.genetics.utah.edu/content/addiction/mouse/)
- For more information of the role of dopamine, reward circuits and the regulation of food intake http://www.cellbiol.net/ste/alpobesity3.php

α- AND β-ADRENOCEPTORS

The receptors that respond to adrenaline or noradrenaline are called adrenoceptors (or adrenergic receptors), and they are present in a wide range of tissues. Pharmacologically they are divided into two major classes, α and β (Table 3-1). Already in 1913, Dale showed that adrenaline had two distinct actions, namely vasoconstriction in certain vascular beds and vasodilation

TABLE 3-1 Adrenoceptor families and properties that distinguish α- and β-receptors

Members of the adrenoceptor family (ADR)	**α**1 A,B,D **α**2 A,B,C	**β**1 **β**2 **β**3
Organ/tissue	**α**	**β**
Sweat/salivary glands	K^+ release (water secretion) (α1)	Salivary amylase secretion (β1)
Arrector pili muscle (hair)	Smooth muscle contraction (α1)	
Eye	Ciliary muscle relaxation (α1) (pupillary dilation)	Radial muscle contract (β_2) (lowering of intraocular pressure)
Bronchial smooth muscle	Minor bronchoconstriction (α1)	Major bronchodilation (β2)
Skeletal muscle	-	Glycogenolysis (β2) Tremor (presynaptic β2) Thermogenesis (β3)
Heart	-	Increased heart contractility (β1) (inotropic effect) Increased heart rate (β1) (chronotropic effect)
Arteriolar smooth muscle	Vasoconstriction in viscera (not liver) and skin (α1, very few α2)	Vasodilation in skeletal musculature and liver (β2)
Uterine smooth muscle (myometrium)	Contraction (α1)	Relaxation (β2)
Liver	Glycogenolysis (α1)	Glycogenolysis (β2)
Adipose tissue	-	Lipolysis (β2,β3) thermogenesis (β3)
Pancreas	Reduced insulin secretion (α_2)	Increased insulin secretion (β2)
Platelets	Aggregation (α2)	-
Adrenergic nerve terminal (presynaptic membrane)	Decrease release (α2)	Increase release (β2)

in others. He showed that the vasoconstrictor component disappeared if the animal was first injected with an ergot derivative. It was later recognized that some ergot alkaloids, in particular, ergotamine and dihydroergotamine, act indeed as selective α-receptor antagonists and by blocking these Dale only found the constrictive effect of β2-receptors (for more information about agonists and antagonists, see textbox "Pharmacological definitions" further

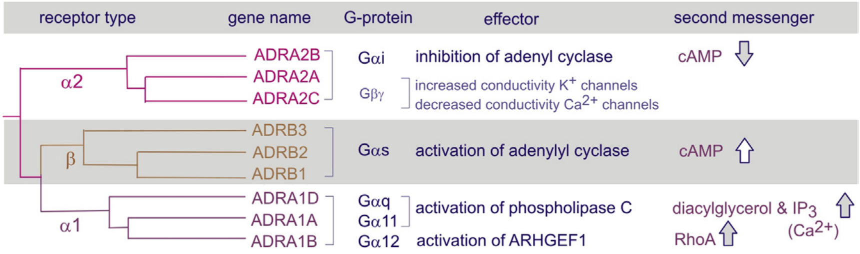


FIGURE 3-3 Adrenoceptor classes and their concomitant signaling mechanisms.

down in the chapter). Starting around 1945, Raymond Ahlquist returned to the question of adrenaline action and concluded that the disparate effects he observed could not be explained by the existence of a single type of receptor. Thus Ahlquist proposed two classes, the α- and β-adrenotropic receptors (Ahlquist, 1948). Curiously, despite sound experimental evidence, Ahlquist himself expressed his doubts about their actual existence. In his own words:

> *this [distinction] would be true if I were so presumptuous as to believe that α and β receptors really exist. There are those that think so and even propose to describe their intimate structure. To me they are an abstract concept conceived to explain observed responses of tissue produced by chemicals of various structures.* ***Ahlquist (1973)***

Nevertheless, his receptor classification gained wide recognition in 1956, shortly after publication of the first edition of Drill's textbook *Pharmacology in Medicine* to which Ahlquist had contributed a chapter about "Adrenergic Pharmacology" (Black, 2010). Much later, with more agonists and antagonists, and importantly with gene sequences at hand, multiple receptors came to light and the initial α- and β-classes were further subdivided (Dixon et al., 1986). Thus, we recognize six α-receptors: α1A, α1B, α1D and α2A, α2B, α2C and three β-receptors, β1, β2, and β3. The pharmacological broad distinction of α- and β-receptors is not fully followed by genetic phylogeny because α1-receptors are closer to β-receptors than they are to α2. Moreover, the distinction does not reflect signaling mechanism as α1-and α2-receptors have quite distinct signaling pathways (Figure 3-3).

All adrenoceptors qualify as GPCR, characterized by seven transmembrane domains (7TM). Starting from the N-terminus, the helices are numbered H1–H7. The short helix at the cytosolic segment, near the C-terminus, is often numbered H8, but it does not traverse the membrane (Figure 3-4). The helices are connected by loops, extracellular ECL1–3, and intracellular ICL1–3. GPCRs have their name because their intracellular loops interact with heterotrimeric G-proteins (GTP-binding proteins composed of three subunits Gα, Gβ, and Gγ). Through specific interactions with Gα subunits, the adrenoceptors are connected to two main effector systems. Class α1-receptors interact with Gαq et Gα11 and these stimulate

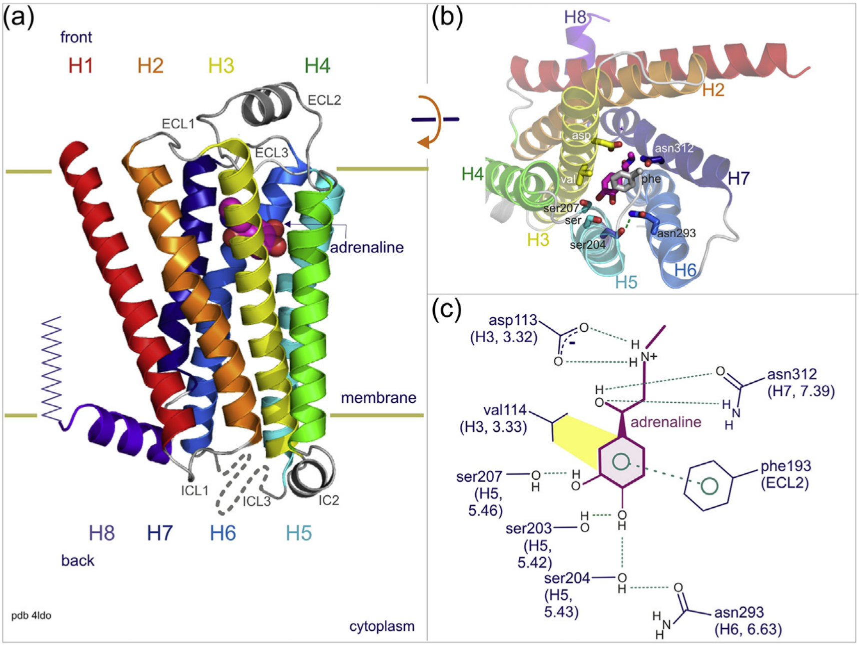


FIGURE 3-4 Adrenoceptor and molecular detail of the adrenaline-binding pocket. (a) The receptor is composed of seven transmembrane helices, H1–H7 colored in rainbow sequence (going from red to dark blue). H8 is a short helix that comprises the cysteine-palmitoylation site. The extracellular (ECL1–3) and intracellular (ICL1–3) are colored light gray. Note the position of adrenaline, nicely buried inside the receptor. (b) The adrenaline-binding pocket as viewed from outside the cell (ECL2 is not represented). Adrenaline is coordinated by a number of hydrogen bonds (ser203, -204, -207), an electrostatic bond (salt bridge) (asp113), one hydrophobic interaction (val114), and a pi/sigma-stacking (phe193). Importantly, adrenaline brings about a change in the side chain rotamer conformation of ser207. This brings about a hydrogen bond between Ser204 and Asn293, thus reinforcing the otherwise weak contact between H5 and H6. As a consequence, the binding pocket contracts as witnessed by a shorter distance, of 1 Å, between asn312 and ser207. Note that H1, H2, and H4 do not take part in binding. Despite the presence of adrenaline, the receptor shown here is not in its fully active state. This requires the presence of the alpha subunit of a G-protein. pdb; 4ldo. (c) Schematic representation of the coordination of adrenaline inside the β2-adrenoceptor. The dotted lines represent hydrogen or electrostatic bonds, the yellow surface a hydrophobic interaction, and the green-dotted line with circles represents pi/sigma stacking. Each amino acid is indicated in two fashions: its number in the sequence (asn312) of the human receptor (ADRB2) and a generic numbering (7.39) according to the proposition by Ballesteros and Weinstein (1995).

PLC, class α2-receptors interact with Gαi and this causes inhibition of adenylyl cyclase and all members of class β interact with Gαs leading to the stimulation of adenylyl cyclase. The Gβγ subunits associated with Gαi also play an important role in signaling, in particular, at the level of presynaptic membranes which often expresses class α2 receptors (retrocontrol). Gβγ subunits interact with- and increase the conductivity of inward rectifying K^+-channels leading to reduced excitability of neurons and they interact with and reduce the conductivity of type-2 voltage-dependent Ca^{2+} channels (Figure 3-3) (Whorton and McKinnon, 2013; Betke et al., 2012). We will return to these effectors in more detail later in the chapter.

Web resource adrenoceptors

Hugo gene nomenclature committee (HGNC)

- Adrenoceptors, alpha (ADRA): http://www.genenames.org/genefamilies/GPCRA#ADR#ADRA
- Adrenoceptors, beta (ADRB): http://www.genenames.org/genefamilies/GPCRA#ADR#ADRB

ADRENALINE-BINDING AND G-PROTEIN-COUPLING MECHANISMS

Adrenaline enters the adrenoceptor from the extracellular phase and docks into a pocket that is buried inside the membrane, surrounded by the transmembrane helices H3, H5, H6, and H7. It makes hydrogen bonds with three serine residues in the H3 helix (ser203, -204, -207), a salt bridge with asp113 in H3, a hydrophobic interaction with val114 in H3 and a pi/sigma stacking with phe193 of the second extracellular loop (ECL2). As we will see later, the multiple contacts with serines are an important feature of its "agonist" characteristic, as it increases the interaction between H5 and H6 through the creation of an additional hydrogen bond between ser204 and asn293 (Figure 3-4). Binding of adrenaline (or its agonist) is translated in two important changes at the cytosolic side of the receptor. The first is an α-helical extension of H5 (it gets 10 amino acids longer) and the second, a 14 Å outward movement of H6. These changes create a G-protein docking site which stabilizes the receptor in its active conformation (Figure 3-5). Without docking of the G-protein, despite the presence of an agonist, adrenoceptors (both α and β) rapidly return, in the order of milliseconds, to an inactive conformation (Rosenbaum et al., 2011).

As we will see later, Gα-subunits are not sole proteins that have the privilege of docking into the pocket of activated adrenoceptors, two other types of protein have been reported to insert into the pocket, namely members of the G protein-coupled receptor kinases (GRKs) and members of the arrestin family.

Within the transmembrane core, the docking of Gαs involves primarily nonpolar interactions. An exception involves pi-stacking of

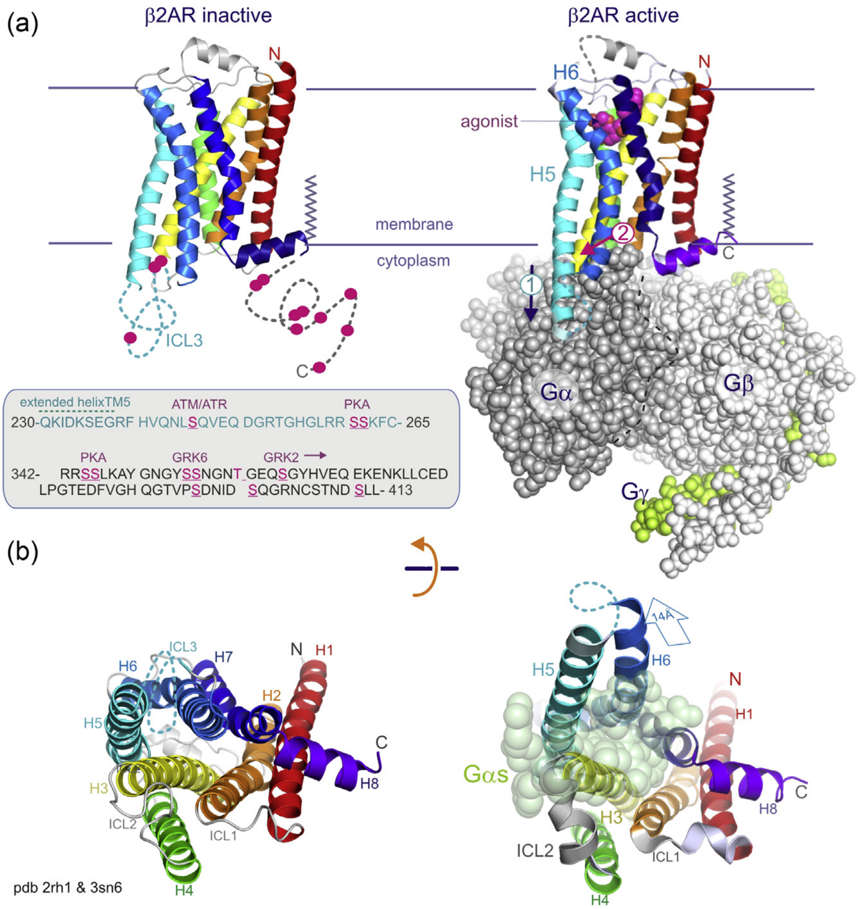


FIGURE 3-5 Conformational changes in the cytosolic segment of the β2-adrenoceptor after binding of agonist. (a) The lateral view on the left shows the characteristic transmembrane-helix structure of an inactive β2-adrenoceptor. The dotted lines represent the long 3rd intracellular loop (ICL3) and the C-terminal domain. These are unstructured and their position cannot be calculated from an X-ray diffraction pattern. The pink spheres are known phosphorylation sites and their position in the sequence, as well as the protein kinase by which they are phosphorylated, are shown in the gray insert. The receptor on the right carries an adrenaline agonist (8-[(1R)-2-{[1,1-dimethyl-2-(2-methylphenyl)ethyl] amino}-1-hydroxyethyl]-5-hydroxy-2H-1,4-benzoxazin-3(4H)-one) and is associated with the Gα-subunit of a heterotrimeric G-protein. Important changes are found in H5, which has an extended helix comprising an additional 10 amino acids (see in insert) as well as a profound outward displacement of H6 (amplitude of 14 Å). (b) Cytoplasmic view (in the plane of the membrane) of the β2-adrenoceptor. On the left, an inactive receptor, on the right an active receptor complexed with GαS. Note the helical extension of the H5 and the 14-Å outward displacement of H6. Together, these changes create a pocket that fits the Gαs, of which only a small section is represented in transparent spheres. The G-protein makes direct contacts with receptor transmembrane helices H3, H5, and with loop ICL2. Pdb: 2rh1, 3sn6.

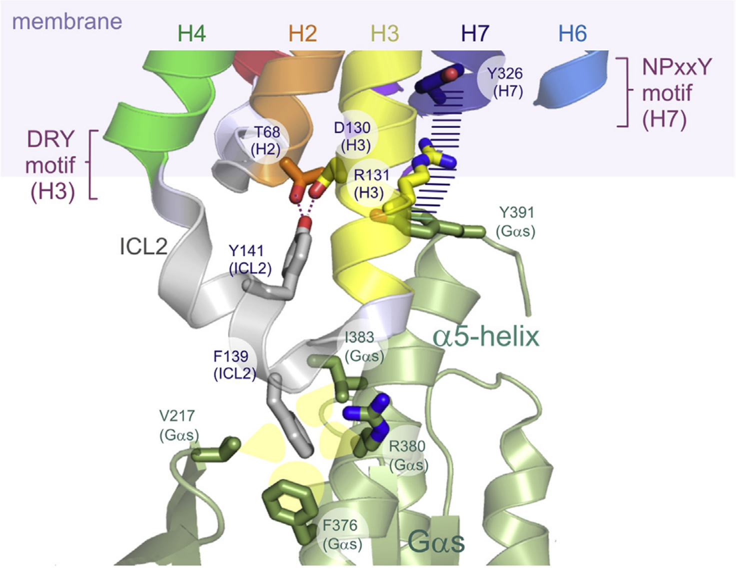


FIGURE 3-6 Major contact sites between Gαs and agonist-bound β2-adrenoceptor. The α5-helix of Gαs docks into a cavity on the intracellular side of the receptor caused by the opening of helices 5 and 6. Within the transmembrane core, the interactions are primarily nonpolar (not shown). An exception is the pi-stacking of tyr326 (receptor H7), arg131 (receptor H3), and tyr391 (Gαs). As the α5-helix exits the cavity, it forms a network of polar interactions with H3 and H5 (not shown). A little further down, the α5-helix of the G-protein forms a hydrophobic pocket (delineated by V217, F376, R380, and I383) which makes contact with phe139 of the intracellular loop ICL2. This interaction is made possible, thanks to the stabilization of ICL2, by hydrogen bonds between tyr141 (ICL2) and asp130 (receptor H3) and thr68 (receptor H2). Both the pi-stacking and the hydrophobic interaction with ICL2 rely on residues that are part of GPCR signature sequences: DRY (providing D130) and NPxxY (providing Y326) respectively. Again, their functional importance provides an explanation for their conservation throughout evolution. H5 and most of H6 of the adrenoceptor are not shown for clarity reasons. Pdb: 3sn6.

tyr391 of the α5-helix (G-protein) against Arg131, in the conserved DRY sequence in H3, and against Tyr326, in the conserved NPxxY sequence in H7 (Figure 3-6). As α5-helix exits the receptor it forms a network of polar interactions with residues in H3 and H5. The second intracellular loop (ICL2) also contacts Gαs via its phe139 which docks into a hydrophobic pocket on the G-protein (composed of val217, phe376, arg380, and iso383). The correct positioning of ICL2 is in part determined by an interaction between tyr141 and asp130, the latter which is situated in the conserved DRY sequence of H3 (Rasmussen et al., 2011). DRY and NPxxY are so-called "signature sequences," they are conserved during evolution and have become a hallmark of GPCRs. Their strategic position in connecting ligand binding with intracellular signaling events explains why. An identical set of interactions occurs in the docking of Gαt to activated rhodopsin (light receptor of the retina) (Scheerer et al., 2008).

G-protein activation

The functional consequence of Gαs binding to the adrenaline-bound receptor is an exchange of GDP for GTP, after all GPRCs are not just receptors, they also are guanine nucleotide-exchange factors (GEFs). The activated receptor engages the C-terminus of the α5-helix of Gαs (Figure 3-6) which undergoes a rigid-body translation upward into the receptor core and as a consequence reorganizes the β6-α5 loop (a region that participates in binding of the purine ring of GTP (not shown)). The intracellular loop, ICL2, also contacts Gαs (for detail see Figure 3-5) and this causes the loss of coordination of the β-phosphate (2nd) and subsequent destabilization of the GDP binding. The contact with ICL2 also disrupts the interaction between two subdomains of the Gαs protein; the GαRas-like domain (GαRas) and the alpha-helical domain (GαAH). This occurs primarily through the loss of contact between R201 and E50 (both in GαRas) and which are shown in cyan spheres in Figure 3-7(a). As a result, GαAH folds away, allowing GDP to freely dissociate. GTP now binds the empty pocket, resulting in reformation of the "closed" conformation. The α-helical domain is unique to heterotrimeric G-proteins, it is absent in all the other "small" G-proteins. It plays a role in stabilizing the GTP binding but also in facilitating its subsequent hydrolysis (intrinsic GTPase activity). The Gαs.GTP subunit functionally dissociates from Gβγ and eventually uncouples from the β2AR (but the timespan is not known). The heterotrimeric G-protein is now said to be "active," ready to interact with downstream effectors, a sequence of events schematized in Figure 3-7(b) (Westfield et al., 2011; Rasmussen et al., 2011; Chung et al., 2011). Thus, with the arrival of adrenaline, a cascade of events is set in train that is generally summarized as: (1) receptor activation by first messenger, (2) recruitment and activation of a transducer (G-protein), and (3) activation of effectors (adenylyl cyclase) followed by production of second messenger (cAMP).

Gβγ subunit also interacts with effectors but in the context of the β2-adrenoceptor signaling, it is not yet clear which effector is mobilized. Typical Gβγ effectors are rectifying K^+ channels (KCNJ3, -6, -5, -9), of which the conductance is increased (Morris and Malbon, 1999), adenyl cyclase type II, -IV (stimulation), N-type voltage-gated Ca^{2+} channels (reduction of conductance), phospholipase A2 and phospholipase Cβ (stimulation), and phosphatidylinositol 3-kinase (stimulation) (Clapham and Neer, 1997). It is generally assumed that members of the Gαi/o-proteins are the

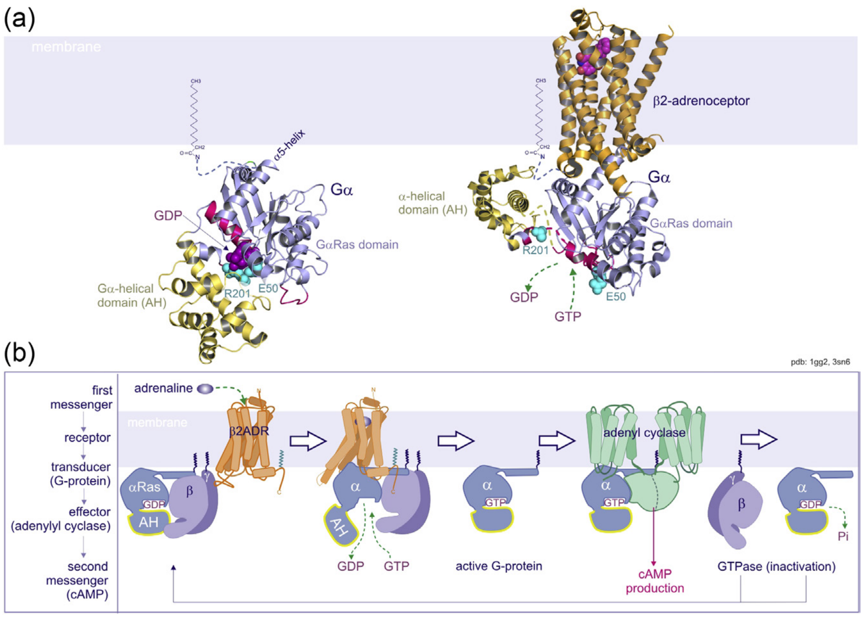


FIGURE 3-7 β2-adrenoceptor-mediated nucleotide exchange on Gαs. (a) The α-subunit of heterotrimeric G-proteins (Gα) is composed of two domains: a GαRas domain and an α-helical domain (AH). The GαRas domain carries the highly conserved guanine-nucleotide coordination sites that are common to all G-proteins (heterotrimeric and monomeric). They are highlighted in pink. The GαRas domain also carries the switch-regions with which the G-protein interacts with downstream signaling components (effectors). The nucleotide-binding pocket is formed by the interface between GαRas and GαAH. Guanine nucleotide binding stabilizes the interaction between these two domains. Upon binding to a ligand-occupied receptor (adrenaline or an agonist), the α5-helix of the Gα-subunit docks into a pocket created by changes in the receptor conformation (structure on the right). This weakens the binding of GDP but it also causes an approximately 127° rotation about the junction between the GαRas ad GαAH domain, leaving the nucleotide fully exposed. Note the loss of contact between R201 and E50, depicted in cyan spheres, which are conserved residues that play a role in the coordination of the guanine nucleotide. It is postulated that both the conformational changes in the GαRas domain and the displacement of the AH-domain serve to facilitate the loss of GDP, which is subsequently replaced by GTP (present at 10-fold higher concentration in the cell and stronger binding to pocket due to the 3rd phosphate). The event is referred to as nucleotide exchange and the ligand-bound receptors act as a guanine exchange factor (GEF). It remains to be studied whether or not the same mechanism applies for other GPCR. The Gβγ subunits, normally associated with Gα.GDP, are omitted from the figure. (b) A schematic representation of the events that follow adrenaline binding to the β2-adrenoceptor. Ligand binding changes the conformation of the receptor, thereby, creating a docking site for the Gα-subunit of a heterotrimeric G-protein (Gαβγ). This leads to changes in the GαRas-domain and an important displacement of the GαAH-domain. Together they results in the loss of GDP. It is postulated that the GαAH-domain returns to its original position upon binding of GTP. The Gα.GTP next detaches from the receptor and from Gβγ and, in this example, interacts with the enzyme adenylyl cyclase. It stimulates the production of the second messenger cAMP. The Gβγ domain can also interact with effectors, certain ion channels, phospholipase A or -C, or phosphatidylinositol kinase, but in the context of the β2-adrenoceptor it is not clear which of these are concerned. After some time, GTP is hydrolyzed (GTPase activity) and the loss of Pi leads to a reassembly of the heterotrimeric G-protein complex. Pdb: 1gg2, 3sn6.

major “source” of Gβγ subunits, meaning that Gβγ effects are most pronounced, most easily detected, in pathways of receptors that interact with Gαi or Gαo. We refer to Chapter 2 for a detailed description of G-proteins, their classification, and their GTPase cycle.

ADRENOCEPTOR AGONISTS, ANTAGONISTS, AND INVERSE AGONISTS

Long before the identity of the different adrenoceptors was revealed, their existence was already hinted to by the employment of chemical compounds that go under the name of agonists and antagonists. Agonists mimic the action of adrenaline, and cause, as tested in isolated tissues or whole animals, dilatation of bronchi, narrowing of certain arteries, or an increased heartbeat. Likewise, switching from physiological responses to biochemical analysis, agonists activate specific G-proteins and set in train the above described signaling events (Figure 3-3 and 3-7). Antagonists normally do nothing by themselves but they prevent (or reduce) the effects of adrenaline or its agonists. We now know that adrenaline agonists and antagonists exert their effect by occupying the adrenaline-binding pocket (compare Figure 3-4 with 3-8), and they act by competition.

Three clinical applications of agonists and antagonists deserve extra attention because they are widespread: the use of α1-agonists in nasal sprays (mainly xylometazoline), β2-agonists in the treatment of asthma (salbutamol, terbutaline, salmetrol, or formoterol), and β1/β2-blockers for cardioprotection (propranolol, timolol, and carvedilol or the more β1-specific atenolol and metopropanol). α1-agonists cause vascular constriction and, as a consequence, reduce the swelling of the mucous epithelium, as well as the production of mucus, and hence they increase the flow of air. β1-agonists cause smooth muscle relaxation in the bronchi and their therapeutic value lies in reducing the symptoms of asthma (wheezing) but they do not interfere with the chronic inflammatory condition that marks this disease. Beta-blockers avoid excessive work of the heart and they prevent arrhythmias. They are very useful because both conditions (excessive work and arrhythmias) are lethal, in particular, when oxygen supply to the heart is limited (as a consequence of the obstruction of coronary arteries or as a consequence of high peripheral resistance). With respect to β2-agonists and β1-antagonists, despite their presumed specificity, they are never used together because there is some “spillover.” Heart failure, therefore, is a contraindication for the use of β2-agonists (which would make the heartbeat faster), and vice versa. Beta-blockers may not be subscribed for those who suffer from asthma (as they may exacerbate bronchial

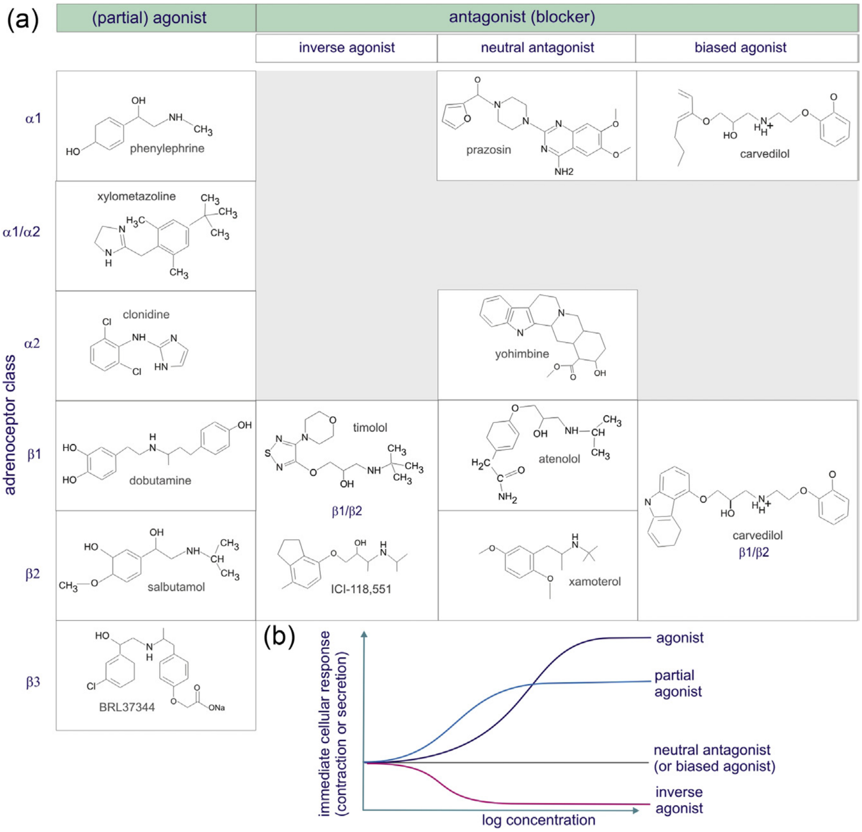


FIGURE 3-8 Structure of some of the adrenoceptor agonists, reverse agonists, biased agonists, and neutral antagonists. (a) The classic pharmacological characterization has recently been turned upside down because of more detailed analysis of downstream events (measurements of activities of different pathways). Agonists stimulate the cellular response and antagonists block the natural ligand-induced response. However, depending on what you measure and under what conditions, they can be further subdivided into inverse agonist, those that inhibit spontaneous activity, neutral agonists, those that block and until today do not provoke any signaling, and biased agonists, those that block the G-protein-mediated events but not GRK/arrestin-induced signaling. Some of these compounds serve as medicament. (b) Graph illustrating the different effects of agonists and antagonists (when measuring the G-protein-coupled events such as muscle contraction or gland secretion).

obstruction). Adrenaline itself is still the favored medication in the case of anaphylaxis (sudden systemic vasodilation due to contact with allergens), in cardiac arrest, and as an adjuvant in the application of local anesthetics, such as lidocaine (local vasoconstriction reduces clearance of the anesthetic and prolongs their efficacy) (Rang et al., 2008).

JAMES BLACK AND THE DISCOVERY OF PROPRANOLOL

Smith and Lawson (1958) had found that hyperbaric oxygen, at 2 atm pressure, reduced the incidence of ventricular fibrillation associated with occlusion of a coronary artery (an experimental method to study angina pectoris). James Black calculated that the oxygen carrying capacity of the blood could only have increased by a maximum of 20% because hemoglobin is already fully saturated with oxygen at the normal atmospheric pressure (1 atm) and free oxygen only dissolves poorly in water. He, therefore, wondered if an equivalently small decrease in the myocardial demand for oxygen could not be just as effective. That was his question. It was well known at the time that the sympathetic nerves instructed heart muscle and other responding cells by the secretion of adrenaline (the role of noradrenaline as a neurotransmitter was still to be discovered). Therefore, he wanted to find a drug that would block the effects of adrenaline on the heart. A major obstacle was the observation that blocking adrenaline leads to an important drop in blood pressure, people felt dizzy when standing up, and this would further deprive the heart of oxygen. In short, treatment with adrenaline blockers would do more harm than good. Black's idea nearly died at birth was it not that he had bought the first edition of Drill's multiauthor textbook of *Pharmacology in Medicine* and came across the chapter by Ahlquist dealing with "adrenergic pharmacology" (published in 1956). In this chapter, Ahlquist proposed the existence of a dual adrenergic receptor system, in which the physiological effects of the catecholamines were mediated by α- and β-receptors. According to this classification, ergot alkaloids qualified as α-receptor antagonists (causing vasodilation) and isoproterenol qualified as a selective activator of β-receptors (affecting heart rate and force). James Black used this information to design his experimental protocol, employing electrically stimulated papillary muscles from guinea pig hearts, in order to develop a β-receptor antagonist and he, and a team of chemists, succeeded. Propranolol reached the market in 1964 under the name of Inderal, the first beta-blocker to give relief to patients with angina pectoris. He was awarded the Nobel Prize in Physiology or Medicine in 1988, shared with Gertrude Elion and George Hitchings, for "discoveries of important principles for drug treatment" (Black, 2010; Black et al., 1964; Black and Leff, 1983; Rubin, 2007).

The initial classification of agonists and antagonists was put into question when new experimental conditions revealed much more diversity in cellular responses to these compounds. Starting with relatively simple definitions of agonists and antagonists, we now recognize partial agonists, inverse agonists, biased agonists, and "neutral" antagonists (see Figure 3-8). A number of substances that qualified as β-adrenoceptor "blockers" (antagonists) were tested in conditions where receptor signaling occurred in the absence of ligand. This is referred to as "spontaneous" or "intrinsic" signaling. Spontaneously signaling is explained by the assumption that GPCR are flexible and can transit, occasionally, from an inactive to active state. In the active state, they interact with G-proteins and cause cellular responses (albeit it very weak or perhaps not reaching a threshold that makes the response noticeable). What agonists (or natural ligands) do is increasing the number of receptors in their active conformation and thus boosting the signaling events. Spontaneous signaling becomes manifest in mice that overexpress the β2-adrenoceptor in the heart. Compared to control animals, isolated hearts of these mice exhibit a high baseline ventricular contractility and a high left atrial tension upon electric stimulation (strong heartbeat). Addition of β-adrenergic agonists does not enhance the contractile force. Surprisingly, addition of ICI-118,551, which qualified as an antagonist, led to a dose-dependent reduction. On the contrary, alprenolol, which also qualified as an antagonist, did what would be expected in the absence of adrenaline; it was without effect. However, when cells were pretreated with alprenolol, the efficacy of ICI-118,551 was greatly diminished (the two compete for the same binding site). It was concluded that ICI-118,551 behaved like an "inverse agonist" (Ehlert, 1986). Alprenolol kept its title of nonselective β-antagonist, as it did not reduce spontaneous signaling but merely acts by blocking agonists and, from now on, blocking inverse agonists too (Bond et al., 1995). Presumed antagonists were also tested in another type of experimental setting where spontaneous signaling occurred, this time through high (ectopic) expression of β2-adrenoceptors in insect Sf9 cells. It was found that spontaneous signaling activity was proportional to the number of receptors expressed (these cells normally do not carry β2-adrenoceptors). Again some antagonists proved to be highly efficient inhibitors of the spontaneous signaling activity as measured by their effect on production of the second messenger cAMP (Chidiac et al., 1993). Among these was timolol and it too qualified as an inverse agonist (Zocher et al., 2012). Spontaneous signaling occurs in other GPCR and inverse agonists have been discovered for histamine- (cetirizine, cimetidine), GABA- (benzodiazepines, barbiturates), 5HT- (chlorpromazine, risperidone), and dopamine receptors (haloperidol, clozapine). The light-receptor rhodopsin makes an exception; it has no basal activity due to the fact that 11-cis-retinal, which is covalently bound to the receptor, acts as a reverse agonist.

Biased agonists

Whereas some antagonists turned out to be inverse agonists, others proved to be biased agonists. Carvedilol, a presumed α/β-blocker, provided additional new insights into receptor functioning. As its qualification indicates, it slows down the heartbeat (β1-blocking action), causes vasodilatation (α1-blocking action), and has a general cardioprotective effect (lower mortality in the follow-up of people who suffered a heart attack), but it does so in a peculiar way. First, when tested in conditions of spontaneous signaling, where cells produce the second messenger cAMP in the absence of adrenaline, it proved to act as an inverse agonist (like timolol and ICI-118,551). The surprise came when it was found that although it inhibited cAMP production, it stimulated a signaling pathway that is normally employed by growth factors; the so-called mitogen-activated protein kinase pathway or MAPK pathway (Galandrin et al., 2008). It was long known that this pathway can be activated by Gαs, but here it occurred under conditions where Gαs is normally not engaged and in fact, it was shown that it even occurred under conditions where the β2-adrenoceptor was mutated in the conserved DRY motif, refraining it from interacting with Gαs (return to Figure 3-6 for detail) (Wisler et al., 2007). Biased agonists are compounds that stimulate only a subset of the signaling pathways that are normally stimulated by agonists or natural ligands. In this respect, to make a clear distinction, classic agonists are sometimes referred to as "balanced-agonists," engaging both Gα-dependent and -independent signaling pathways. The binding of carvedilol to the β1-ADR is shown in Figure 3-10(c) and its interactions differ from a (full) agonist and an inverse agonist. It establishes the ionic interaction with asn329 (H7), hydrogen bonds with asp121 (H3) and ser211 (H5) and it causes a shift in rotamer side-chain conformation of ser212 which, as a consequence, makes a hydrogen bond with asn310 (H6). These are features of an agonist. It makes an extra hydrophobic interaction, both with valine122 and val125. How these differences in ligand binding translate into conformational changes and subsequent interactions with downstream mediators is still a work in progress.

One receptor with multiple conformations

The current line of thinking is that adrenoceptors, like numerous other seven transmembrane receptors (7TM), are rather flexible structures and exist in different conformations. Some of these are active, meaning they allow interaction with downstream components, others are inactive conformations. From an energetic point of view, in the absence of ligand, the inactive conformations are more economical and at any given time most of the receptors linger in the inactive state (o nly some spontaneous activity occurs). Ligands, be them agonists or inverse agonists, change the energy landscape and favor certain conformations and not others (Kahsai et al., 2011). In the case of agonists, these changes create docking sites for cytosolic proteins and their attachment maintains the receptor in a stable active state. Thus it was calculated that docking of Gαs is required to keep the β2-adrenoreceptor in its active conformation (Figure 3-9) (Nygaard et al., 2013). Importantly, it has been shown that members of the GRKs and arrestins too can dock directly onto agonist-bound

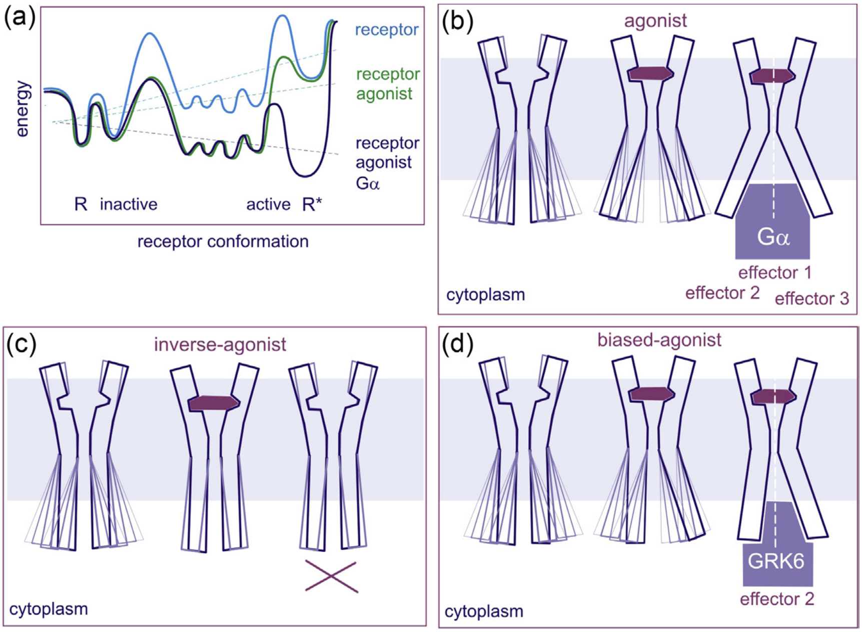


FIGURE 3-9 Proposed energy landscape of the β-adrenoceptor. (a) The energy barriers are sufficiently low to transit from one state into another at body temperature and this explains why spontaneous or intrinsic receptor signaling occurs. However, reaching the active state is an uphill struggle and the incidence will be low. Agonists reduce the threshold of the path toward the active conformation, they reduce the free energy state of the intermediate conformations and they reduce the free energy state of the active conformation. Collectively they increase the probability that receptors reach the active conformation but despite all this, they will not remain there for a long time (calculated to be in the range of milliseconds). Real changes in distribution occur when the agonist-bound receptor teams up with the Gα subunit of G-proteins. The free energy of the active conformation is now sufficiently low in order to "trap" ligand-bound receptors in their active state (R*). Inverse agonists prevent all this and thus reduce the probability of spontaneous signaling. Naturally, their presence will also block access of agonists (or antagonists). In this context, we define antagonists as substances that do not alter the free energy states, they are said to be "neutral" and act by blocking access of agonists (or inverse agonists). (b) A schematic representation of receptor flexibility (based on in silico modeling). Agonists do not hold the receptor in an active conformation, certain flexibility remains. Stabilization in an active conformation only occurs upon docking Gαs. (c) inverse agonists stabilize the receptor in an inactive conformation, meaning that they prevent interacting with G-proteins (or other receptor-docking proteins such as GRKs and arrestins). (d) A speculative receptor-activation scenario, where a biased agonist brings about conformational changes that allow docking and stabilization by GRK6 but not Gαs. Only a limited set of effectors are mobilized, hence a "biased" response. *Images (a) and (b) adapted from Nygaard et al. (2013).*

receptors and initiate Gα-independent signaling events (Huang and Tesmer, 2011). Biased agonists may thus induce selective coupling to only one portion of potential downstream signaling components, that is, omitting Gα but not others, implying that it must induce and/or stabilize a receptor conformation that is distinct from the one induced by a "balanced" agonist.

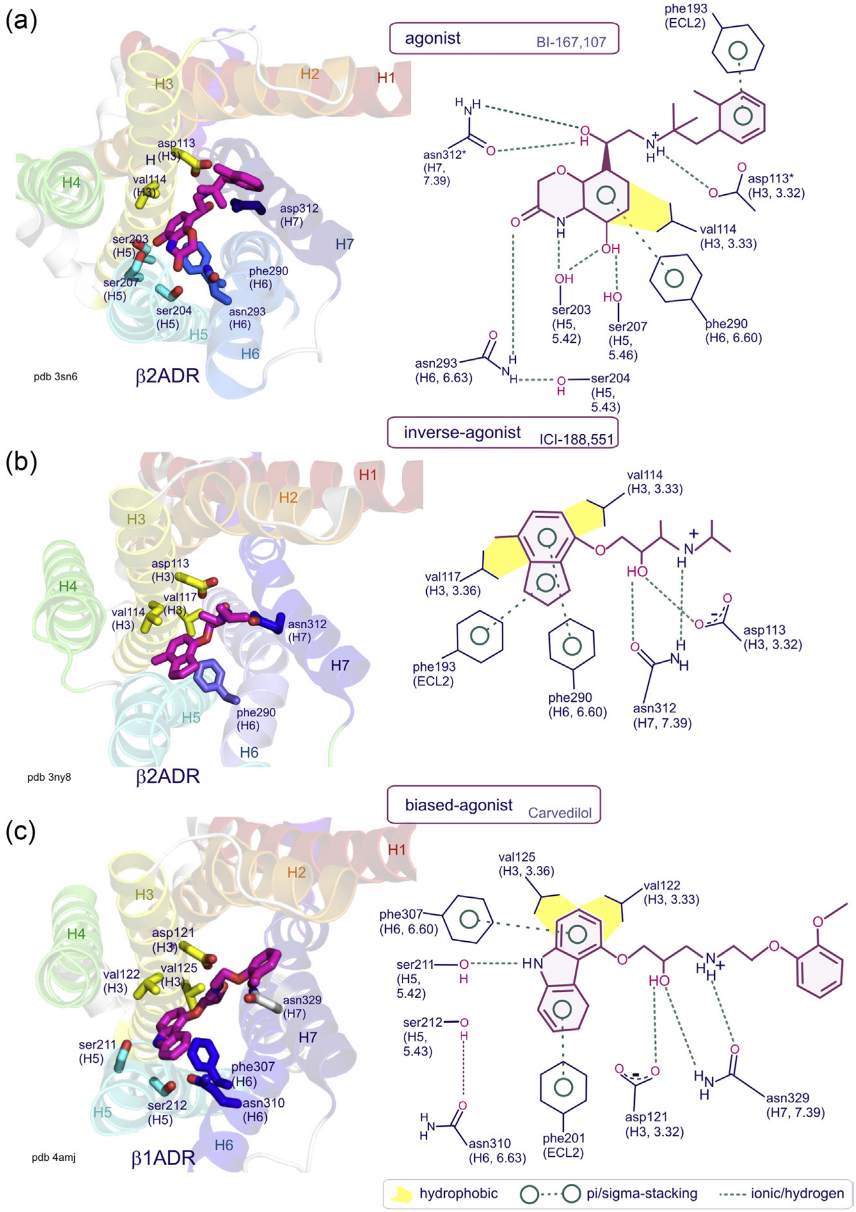
In a simple conceptual scheme, a receptor in unliganded state can be visualized as a continuum of conformations (Dror et al., 2009). Binding of a given ligand likely enriches one of these conformations by shifting the conformational equilibrium, thereby, leading to a specific cellular response. Indeed, evidence of ligand-specific conformations of β-adrenoceptors is forthcoming (Liu et al., 2012; Kahsai et al., 2011). After the section about PLC we will return to the signaling events downstream of the adrenoceptors (Gαs-, Gαq-, GRK-, and arrestin-mediated pathways).

HOW DO LIGAND-BINDING CHARACTERISTICS TRANSLATE INTO SIGNALING EFFECTS?

With detailed molecular descriptions at hand, of how an agonist turns on the β2-adrenaline receptor and how this leads to interaction with the Gα-subunit of G-proteins, the question arises what exactly distinguishes agonists from biased agonists, antagonists, or inverse agonists? A comparative study has offered some guidelines but novel findings have indicated that matters are more complex than anticipated due to the fact that the seven transmembrane receptors are rather flexible. With respect to agonists and inverse agonists, compared with the binding of adrenaline (see Figure 3-10 and compare with Figure 3-4), the strong agonist BI-167,107 has similar ionic interactions (salt bridges) and hydrogen bonds with asn113 in H3, two serines in H5, and asp312 in H7. Moreover, the agonist makes hydrophobic and pi-stacking interactions with the receptor. The network of hydrogen bonds with serines on H5 brings about, through a rotamer conformational change, a hydrogen bond between ser204 on H5 and asn293 on H6. As a

FIGURE 3-10 (a) Agonists generally make strong contacts, through ionic interactions (salt bridges) and hydrogen bonds, with H3, H5, and H7. In addition, there are hydrophobic contacts with H3 (val114) and pi-stacking with H6 (phe290) and the extracellular loop-2 (phe193, not shown). The contact with the serines of H5 (ser203, ser207) plays an important role as they promote the interaction of Ser204 with asn293 on H6. This apparently causes a slight contraction of the binding pocket, a strengthening of the interaction between H5 and H6 and a weakening of interactions between H3 and H4. All this is translated in conformational changes at the cytosolic side, such as extension of H5 and an outward movement of H6. In the given example, the agonist is BI-167,107 bound to the human b2-ADR. pdb: 3sn6. (b) Inverse agonists generally exhibit the ionic interactions (salt bridges) with H3 (asp113) and H7 (asn312) but lack one or two hydrogen bonds with H5 ser203 and/or ser207. As a consequence H5 and H6 remain weakly connected. Inverse agonists tend to have better developed hydrophobic interactions and this may help to hold the receptor in an inactive state. In the given example, the inverse agonist is ICI-188,551 bound to the human β2-ADR. pdb: 3ny8, (c) The biased agonist exhibits the same ionic interactions (salt bridges) with H3 (asp212) and H7 (asn329) but it establishes only one hydrogen bond with H5 (ser211). This nevertheless causes a rotamer side-chain conformational change in ser212 (H5) allowing a hydrogen to bind with H6 (asn310). As for the agonist, this must cause a slight contraction of the binding pocket and a strengthening of the interaction between H5 and H6. The biased agonist too has an additional hydrophobic interaction with val125 (compared to the agonist). All this is translated in conformational changes that are different from the agonist as they do not give rise to Gα-mediated signaling but favors a GRK6/β-arrestin-mediated pathway. In the given example, the biased agonist is carvedilol bound to the turkey β1-ADR. pdb: 4amj.

▶


(a)
agonist BI-167,107
H1
H2
H3
H4
H5
H6
H7
asp113 (H3)
val114 (H3)
asp312 (H7)
ser203 (H5)
ser207 (H5)
ser204 (H5)
phe290 (H6)
asn293 (H6)
pdb 3sn6
β2ADR
phe193 (ECL2)
asn312* (H7, 7.39)
asp113* (H3, 3.32)
val114 (H3, 3.33)
ser203 (H5, 5.42)
ser207 (H5, 5.46)
phe290 (H6, 6.60)
asn293 (H6, 6.63)
ser204 (H5, 5.43)
(b)
inverse-agonist ICI-188,551
H1
H2
H3
H4
H5
H6
H7
asp113 (H3)
val117 (H3)
val114 (H3)
asn312 (H7)
phe290 (H6)
pdb 3ny8
β2ADR
val114 (H3, 3.33)
val117 (H3, 3.36)
phe193 (ECL2)
phe290 (H6, 6.60)
asn312 (H7, 7.39)
asp113 (H3, 3.32)
(c)
biased-agonist Carvedilol
H1
H2
H3
H4
H5
H6
H7
asp121 (H3)
val122 (H3)
val125 (H3)
asn329 (H7)
ser211 (H5)
ser212 (H5)
phe307 (H6)
asn310 (H6)
pdb 4amj
β1ADR
val125 (H3, 3.36)
val122 (H3, 3.33)
phe307 (H6, 6.60)
ser211 (H5, 5.42)
ser212 (H5, 5.43)
asn310 (H6, 6.63)
phe201 (ECL2)
asp121 (H3, 3.32)
asn329 (H7, 7.39)
hydrophobic
pi/sigma-stacking
ionic/hydrogen

consequence, the ligand-binding pocket is stabilized in a contracted state as has been observed for adrenaline (the distance between asp312 and ser203 is reduced by 1 Å (Warne et al., 2011). The inverse agonist exhibits the hydrogen bond and salt bridge with asp113 and asn312, respectively, but it does not establish the network of hydrogen bonds with serines on H5 and it has at least one additional hydrophobic interaction (val117 in H3). Somehow, these differences prevent the receptor of adopting an active conformation and, as a consequence, spontaneous signaling is reduced. The biased agonist carvedilol differs from agonists by establishing only one hydrogen bond with H5 and by an additional hydrophobic interaction. Remember, antagonists are somewhere in between, they do not contract the ligand-binding pocket, like agonists do, nor do they prevent the active conformation, like inverse agonists do. The jury is still what this all really means.

NOBEL PRIZE FOR THE DISCOVERY AND STRUCTURE ANALYSIS OF THE ADRENOCEPTORS

Robert Lefkowitz and Brian Kobilka were jointly awarded the Nobel Prize in Chemistry in 2012 "for studies of GPCR." The β2-adrenoreceptor played a remarkable role in elucidating signaling events downstream of seven membrane receptors. It was one of the first seven-transmembrane spanning receptors to be characterized by radioligand binding, it was one of the first neurotransmitter receptors to be cloned (Dixon & et al., 1986) and to be structurally determined by crystallography. The above-mentioned laureates played key roles in all this. For more information: http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2012/.

Online resource to view two-dimensional diagrams of proteins with known structure complexed with ligands. Center of bioinformatics, University of Hamburg, Germany http://poseview.zbh.uni-hamburg.de/.

PHARMACOLOGICAL DEFINITIONS

Agonist. A ligand that binds to and activates a receptor and elicits a physiological response. The endogenous agonist for the β1AR is noradrenaline, which is a full agonist that elicits the maximal response for the receptor in activating a G-protein.

PHARMACOLOGICAL DEFINITIONS *(cont'd)*

Constitutive, spontaneous, or basal activity. Physiological response that occurs in the absence of any receptor-activating ligand due to a fraction of the receptor being in an activating conformation.

Inverse agonist. A ligand that binds to a receptor and inhibits or eliminates, in the case of a full inverse agonist, the basal or constitutive activity of a receptor.

Partial agonist or partial inverse agonist. Ligands that elicit only a partial response when compared to either a full agonist or a full inverse agonist, respectively.

Antagonist. Any ligand that blocks binding of endogenous agonists to the receptor, thereby, modulating receptor activity. A general term that encompasses ligands that may be inverse agonists, partial agonists, or neutral in effect.

Beta-blockers. Term for antagonists of β-adrenoceptors when used in a therapeutic setting.

Biased agonist. A ligand that binds to a receptor and signals to a variable extent through both G-protein-dependent and G-protein-independent pathways. A ligand may be "weakly biased," that is, signaling is mainly via G-proteins or "perfectly biased," that is, the ligand is an inverse agonist of the G-protein-coupled pathway as well as an agonist of the G-protein-independent pathways.

Note. The categorization of ligands can be dependent on the type of assay used, the cell type in which the receptor has been expressed, expression levels, and the sensitivity and dynamic range of the assay used to detect downstream signaling events.

FOR THOSE WHO WISH TO EXPLORE THE SUBJECT FURTHER, BELOW WE SUGGEST SOME KEY ARTICLES.

- Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318, 1258–1265.
- Wacker, D., Fenalti, G., Brown, M.A., Katritch, V., Abagyan, R., Cherezov, V., Stevens, R.C., 2010. Conserved binding mode of human *β*2-adrenergic receptor inverse agonists and antagonists revealed by crystallography. J Am Chem Soc 132, 11,443–11,445.

continued

FOR THOSE WHO WISH TO EXPLORE THE SUBJECT FURTHER, BELOW WE SUGGEST SOME KEY ARTICLES. *(cont'd)*

- Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F.2., Babu, M.M., 2013. Molecular signatures of G-protein-coupled receptors. Nature 494, 185–194.
- Warne, T., Moukhametzianov, R., Baker, J.G., Hehmé, R., Edwards, P.C., Leslie, A.G.W., Schertler, F.X., Tate, C.G., 2011. The structural basis for agonist and partial agonist action on a *β*1-adrenergic receptor. Nature 469, 241–244.
- Rosenbaum, D.M., Zhang, C., Lyons, J.A., Holl, R., Aragao, D., Arlow, D., Rasmussen, S.G.F., Choi, H-J., DeVree, B.T., Sunahara, R.K., Chae, P.S., Gellman, S.H., Dror, R.O., Shaw, D.E., Weis, W.I., Caffrey, M., Gmeiner, P., Kobilka, B.K., 2011. Structure and function of an irreversible agonist-*β*2 adrenoceptor complex. Nature 469, 236–240.
- Nygaard, R., Zou, Y., Dror, R.O., Mildorf, T.J., Arlow, D.H., et al., 2013. The dynamic process of *β*2-adrenergic receptor activation. Cell 152, 532–542.
- Reiter, E., Ahn, S., Shukla, A.K., Lefkowitz, R.J., 2012. Molecular mechanism of *β*-arrestin-biased agonism at seven-transmembrane receptors. Annu Rev Pharmacol Toxicol 52, 179–197.
- Gurevich, V.V., Gurevich, E.V., Structural determinants of arrestin functions. Progress in Mol Biol Transl Sci; 118, 57–92.
- Warne, T., Edwards, P.C., Leslie, A.G.W., Tate, C.G., 2012. Crystal structures of a stabilized *β*1-adrenoceptor bound to the biased agonists bucindolol and carvedilol. Structure 20, 841–849.
- Ring, A.M., Manglik, A., Kruse, A.C., Enos, M.D., Weis, W.I., Garcia, K.C., Kobilka, B.K., 2013, Adrenaline-activated structure of b2-adrenoceptor stabilized by an engineered nanobody. Nature 502, 575–581.

ADENYLYL CYCLASE

Cyclic adenosine monophosphate (cAMP): the first second messenger

Looking back to the time when most wisdom in mammalian biochemistry was derived from experiments using rat liver (perfusions, slices, homogenates etc.), one gets a sense of happy circumstance that led Sutherland to use liver from dog in his investigations of hormonal activation of glycogenolysis (Rall et al., 1957). During the growth and maturation of rats (6–60 days), the expression of β-adrenoceptors in the liver declines while that of the $\alpha 1$-adrenoceptors increases (Nakamura et al., 1984). Adrenergic stimulation of glycogen breakdown in the liver of adult rats is, therefore, mediated primarily through the α-receptors, which induce an elevation in the concentration of cytosol Ca^{2+}. Using dog, Sutherland showed in 1957 that the breakdown of glycogen, in response to adrenaline or glucagon, is consequent on the generation of a heat-stable and dialyzable factor (Rall et al., 1957). This factor proved to be cyclic AMP, the first second messenger to be identified.

Although cyclic AMP (henceforth cAMP) is most famously the intracellular signal for the breakdown of liver glycogen (and correspondingly for the shutdown of glycogen synthesis), it is a key mediator for many other important receptor responses. We return to these questions in the chapter about signaling events involved in long-term potentiation (Chapter 7 "Bringing the signal into the Nucleus: Regulation of Gene Expression"). cAMP is also present in prokaryotes, both Eubacteria and Archaea, and plants, the other fundamental domains of life (Leightling et al., 1986; Bolwell and Cyclic, 1995). Although its functions as a regulator in nonnucleated cells (prokaryotes) are very different from those of eukaryotes, its generation has signified a response to starvation throughout evolution. cAMP is synthesized in *Escherichia coli* starved of glucose and it induces expression of other sugar-metabolizing enzymes such as β-galactosidase. In this case, it interacts with promoter elements in the vicinity of the RNA polymerase site and is directly involved in the regulation of gene transcription (Gorke and Stulke, 2008). In yeast (unicellular, eukaryotic), cAMP is a growth signal. The presence of nutrients causes an increase in intracellular cAMP, whereas in starvation conditions the cellular content of cAMP declines, growth ceases, and the cells switch to meiosis and spore formation (Busti et al., 2010). In the slime mold *Dictyostelium discoideum* under conditions of starvation, cAMP is secreted into the environment by the individual cells as a chemotactic signal for assemblage into a slug, leading to subsequent spore formation (Darmon et al., 1975). Although it has generally been hard to discern functions for cAMP in plants, there is now good evidence that it acts as a regulator of ion channels for both K^+ and Ca^{2+}. The K^+ channels in the guard cells of the leaves of *Vicia faba* (broad bean) appear to be regulated by a cAMP (Li et al., 1994).

cAMP is formed from ATP

cAMP (strictly, 3′,5′-cyclic adenosine monophosphate) is synthesized from ATP which is present in all cells at high concentration (5–10 mmol/l). The formation of cAMP is catalyzed by adenylyl cyclases and its removal, by conversion to 5′-AMP (adenylate), is catalyzed by the action of phosphodiesterases (cAMP-PDE) (Figure 3-11). Adenylyl cyclase facilitates the transfer of an electron, from oxygen on the ribose ring, to the first phosphate (alpha position). The result is a cyclic bond between carbon 3 and 5, hence the name 3′,5′-cyclic AMP (or cAMP), and the liberation of PPi.

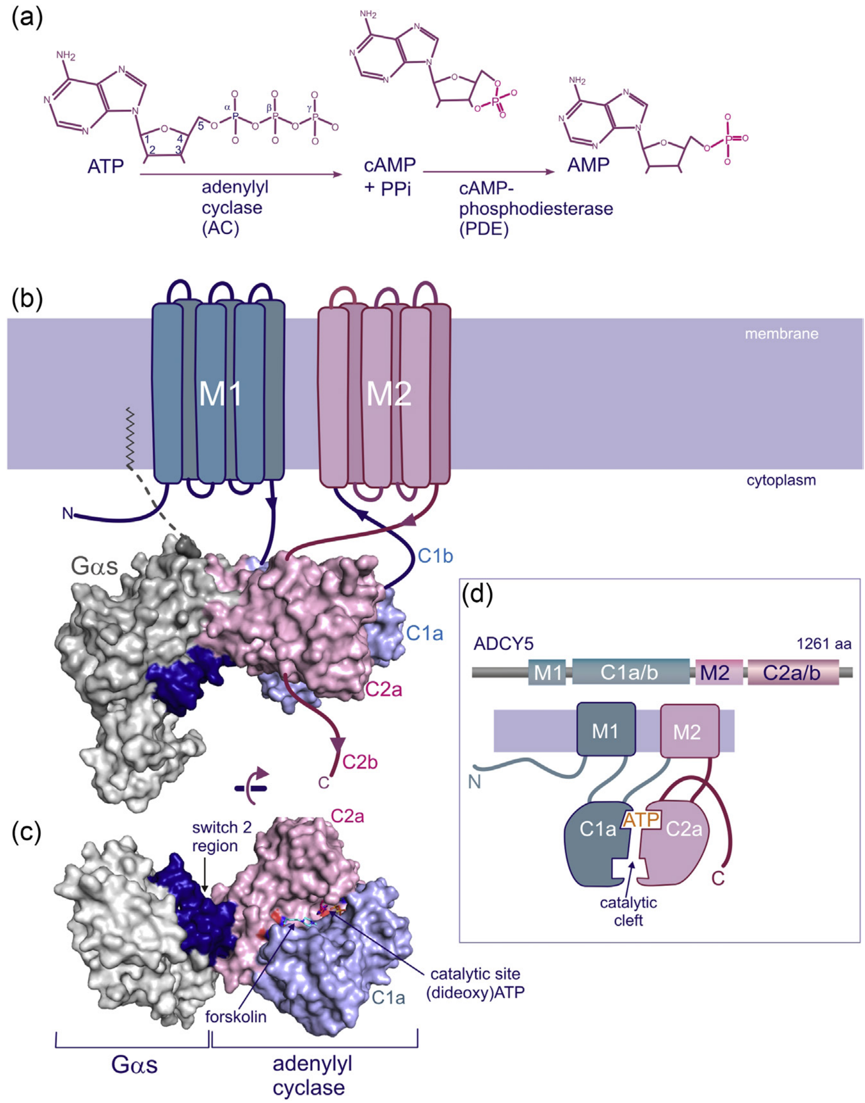


FIGURE 3-11 Domain architecture and topology of adenylyl cyclase. (a) Adenylyl cyclase converts ATP into cyclic AMP (second messenger) and inorganic bisphosphate (PPi). The phosphodiesterase (cAMP-PDE) converts cyclic AMP into AMP (no longer active in the signaling pathway). (b) Schematic representation of the transmembrane domains (M1 and M2) and realistic surface representation ("side view") of the catalytic dimer (C1a/C2a) of adenylyl cyclase. The enzyme is composed of two membranes (M1, M2) and two cytosolic domains (C1a/b, C2a/b). The cytosolic domains associate as an asymmetric dimer and the interface between the two (C1a/C2a) constitutes the catalytic cleft with one ATP-binding site. The catalytic subunit shown here is bound to Gαs (in gray) of which the switch-2 region is colored dark blue. The domain C1b is shown as a string connecting M2 with C1a and domain C2b as a string that constitutes the C-terminal segment. (c) The "bottom view" of adenylyl cyclase/Gαs reveals how the switch-2 region of Gαs (dark blue) inserts into C2a (and does not contact C1a). The catalytic cleft is occupied by forskolin, a potent stimulant, and by dideoxy ATP, a non hydrolyzable analog for adenylyl cyclase (because it lacks the 3-hydroxyl group on the ribose). The ATP-binding site is sometimes referred to as the P-site, for purine-binding site. Pdb; 1cjk. (d) A schematic representation to illustrate the relationship between domain architecture of adenylyl cyclase and the structural organization of the enzyme.

The enzyme requires a conformational change which enables the correct positioning of a number of amino acids in order to coordinate ATP and two metal ions (which help to make ATP a strong nucleophile) (Figure 3-12).

Structural organization of adenylyl cyclases and their regulation by G-proteins

Adenylyl cyclase remained an activity without a proper molecular description for about 30 years, until 1989. At first, the possibility was entertained that more than one enzyme might be involved in the cyclization reaction. It is the importance of adenylyl cyclase in metabolic regulation and the simplicity of the reaction it catalyzes, that provoked the intense investigation of its properties. A major problem for research in this area was the fact that adenylyl cyclase, an integral membrane protein, generally comprises not more than 0.01–0.001% of total membrane protein (an exception is the olfactory epithelium in which it comprises more than 0.1%). The poor solubility of membrane proteins in aqueous media and their dependence upon the phospholipid environment for the maintenance of their tertiary structure, make them difficult to isolate, purify, and reconstitute into a form upon which sensible measurements can be made. Nevertheless, the G_s-coupled form of adenylyl cyclase, present in many tissues, and a brain form of the enzyme have been purified and these provided sequence information that paved the way to cloning of multiple genes. Indeed, the first cDNA encoding a mammalian adenylyl cyclase revealed a protein of structural complexity commensurate with the difficulties encountered in its purification and expression (Krupinski et al., 1989).

> *The preparation of adenyl cyclase in a simplified and purified form has been hampered by the association with particulate material in the "*nuclear*" fraction, by its lability and by its close association with the detergent Triton after solubilization. It is hoped that the enzyme may be solubilized by other procedures and that more stable soluble preparations can be achieved.* ***Earl Sutherland et al. (1962)***

It is simple now to measure the level of cAMP with the use of kits, binding proteins, and specific antibodies. Not so simple for Sutherland and his colleagues who measured the product cAMP using a two-stage assay procedure. The first involved incubation together with ATP, glycogen phosphorylase b, and fractions of dog liver homogenate (containing phosphorylase kinase and protein kinase A, PKA). This was followed up by measurement of the activity of the active product phosphorylase a on its substrate glycogen (Sutherland et al., 1962; Sutherland, 1972). Earl W Sutherland (1915–1974) was awarded the Nobel Prize in Physiology of Medicine in 1971 "for his discoveries concerning the mechanisms of action of hormones."

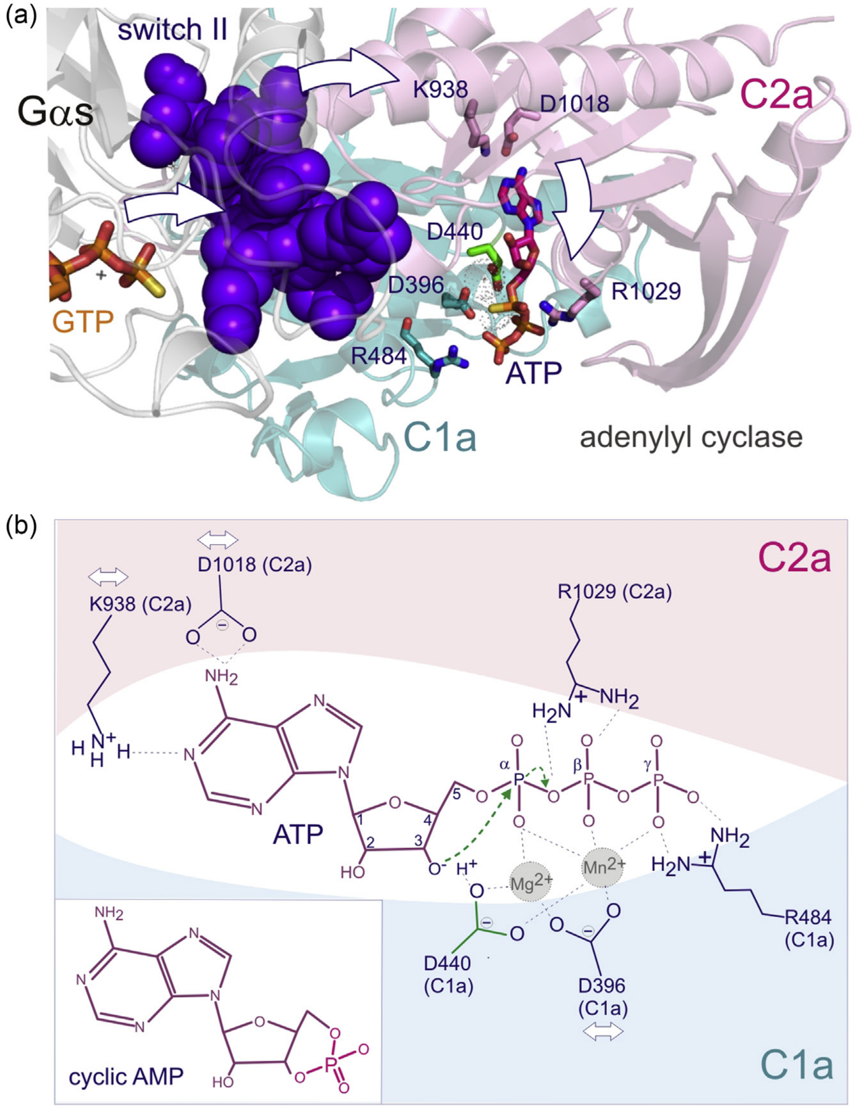


FIGURE 3-12 Cartoon representation of catalytic cleft of adenylyl cyclase. Binding of Gαs sets in train a series of conformational changes that lead to hydrolysis of the phosphoanhydride bond between the α- and β-phosphate. The cascade starts with binding of GTP to Gαs and subsequent changes in the switch regions, among which switch-2 (represented by purple–blue spheres). This leads to functional dissociation with Gβγ and binding to the C2a subunit of adenylyl cyclase. Among other events, the binding leads to changes in the position of K938 and D1018, both of which coordinate the purine rings of ATP. It also causes a movement of D396, which together with D440, coordinates the metal ions (shown as dotted spheres) and renders the 3-hydroxy of the ribose more nucleophile. Two arginines, R484 and R1029, play a role in negative-charge compensation during the reaction. The rearrangement initiates the nucleophilic attack on β-phosphate (green-dotted arrow), leading to the generation of cAMP and PPi. The white arrows roughly indicate the set of motions that bring adenylyl cyclase in its competent (active) state. Pdb; 1cjk.

Adenylyl cyclase, with the exception of one soluble variant (ADCY10), is a 12-membrane spanning protein, probably a fusion of two identical ATP-binding membrane proteins (Figure 3-11). The amino acid sequence reveals sequence similarity with ATP-driven membrane pumps and with the cystic fibrosis chloride conductance channel. The ATP-binding domains (C1a/C2a) form an asymmetric dimer of which the interface contributes to binding of ATP and catalysis. In the example shown, of the two theoretical ATP-binding pockets, only one is functional. The other pocket is degenerated but was shown to bind forskolin, a labdane diterpene that is produced by the Indian Coleus plant (*Coleus forskohlii*) and acts as a strong (but artificial) stimulant. It should be noted that this is not true for all adenylyl cyclases, some members carry two functional catalytic sites. Adenylyl cyclase provides two symmetrically related binding sites for the G-protein subunits Gαs (stimulatory) and Gαi (inhibitory) (Tesmer et al., 1997b; Zhang et al., 1997). Again, the soluble ADCY10 forms an exception; it is not regulated by G-proteins and is not stimulated by forskolin.

The reaction catalyzed by adenylyl cyclase is similar to that of DNA polymerases. Both enzymes cause the hydrolysis of the phosphoanhydride bond between the α- and β-phosphate of a nucleoside triphosphate. Despite no obvious sequence homology, the enzymes do possess similar domains. The similarity has helped to elucidate the structure–function relationship of the adenylyl cyclase (Artymiuk et al., 1997). Of the four known families of DNA polymerases, three have a core “palm” domain composed of a βαββαβ motif (α-helices, β-strands). At distinct topological locations within this motif are two invariant aspartic acid residues (D396, D440 in Figure 3-12) that coordinate two metal ions (Mn^{2+} or Mg^{2+}). The metal ions serve to render the α- and β-phosphates electrophilic, whereas D396 serves to render the 3-hydroxyl nucleophilic (by attracting a proton). As a consequence, under the appropriate conditions, the phosphoanhydride bond is broken between α- and β-phosphate, resulting in a cyclic bond of the α-phosphate with 3- and 5-hydroxyl (hence cyclic AMP) and the liberation of inorganic diphosphate (PPi). Regulation of activity of adenylyl cyclase constitutes the correct positioning of a number of key residues (see Figure 3-12). Active Gαs, in which binding of GTP has led to changes in conformation of the switch regions, binds to C2a and this renders the enzyme competent through a cascade of conformational changes that cause the correct positioning of residues important in the coordination of both ATP and the metal ions, and the correct positioning of the catalytic residue D396 (Hurley, 1999).

Other regulators of adenylyl cyclase

The enzyme is inhibited by GαI, which binds C1a in a fashion similar as Gαs binds to C2a, but fixes the enzyme in an incompetent (inactive) state. Ca^{2+}/calmodulin docks onto C2a and renders the enzyme active, as does Gβγ (but binds at a different site) (Hurley, 1999; Diel et al., 2008). Phosphorylation by PKA causes inhibition, whereas phosphorylation by protein kinase C causes activation. Elevated levels of cytosolic free calcium compete with metal-ion binding in the case of ACDY5 and -6 and so inhibit the catalytic process. In the case of ACDY10, the soluble variant, elevated levels of Ca^{2+} stimulate catalysis (Mou et al., 2009; Tresguerres et al., 2011). Dideoxypurines act as inhibitors; they bind the adenosine-binding pocket (contacts with K938 and D1018) but cannot be processed by the enzyme (Tesmer et al., 2000). Finally, forskolin binds into the catalytic cleft and enhances catalytic activity both in the absence and presence of Gαs (Linder and Schultz, 2008).

Adenyl cyclase as coincidence detector

Because of the multiple sites of regulation of adenylyl cyclase, different signals can be integrated on one enzyme. For instance, ADCY2, -4, and -7 are stimulated both by Gαs and Gβγ. The origin of the Gβγ subunits is not necessarily from Gs, they may also originate from Gi or Go. Members of Gαi inhibit ADCY1, -5, -6, or -9 but their corresponding Gβγ may boost the activity of the aforementioned adenylyl cyclases.

ANTHRAX EDEMA FACTOR IS A SOLUBLE ADENYLYL CYCLASE.

Bacillus anthracis, the etiologic agent for anthrax, secretes edema factor (EF) to disrupt intracellular signaling pathways. Upon translocation into host cells and association with a calcium sensor, calmodulin (CaM), EF becomes a highly active adenylyl cyclase that raises the intracellular concentration of cAMP. Growing evidence shows that EF plays a key role in anthrax pathogenesis by affecting cellular functions vital for host defense. This strategy is also used by *Bordetella pertussis*, a bacterium that causes whooping cough. Pertussis bacteria secrete the bifunctional toxin CyaA, which after cleavage in hemolysin and adenylyl cyclase, raises the intracellular cAMP levels (in a Ca^{2+}/calmodulin-dependent manner). CyaA should not be confused with pertussis toxin (PTX), another virulence factor from *Bordetella pertussis* (Tang and Guo, 2009).

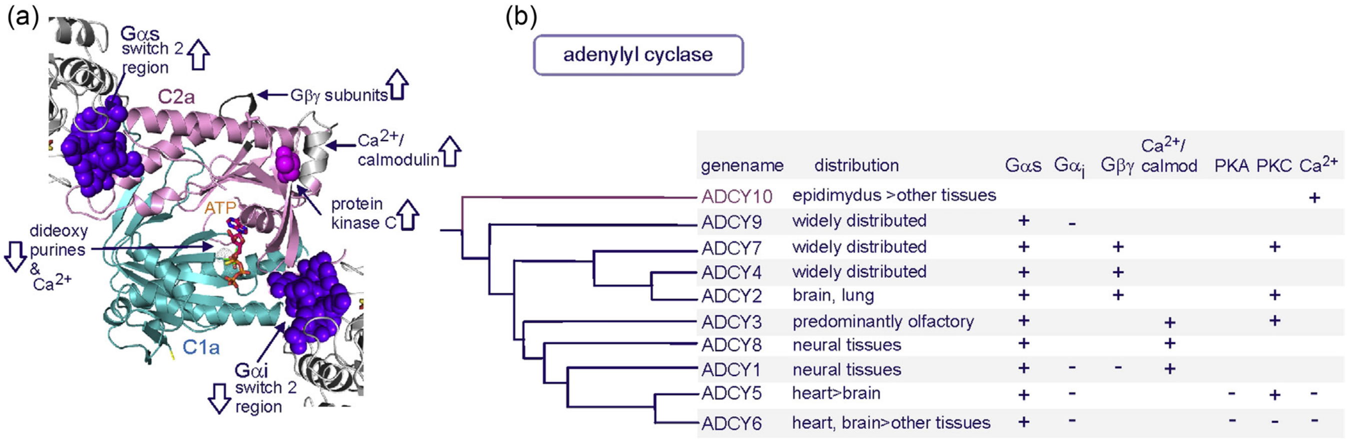
CHOLERA AND PERTUSSIS TOXIN

Pertussis toxin (PTX) is a virulence factor secreted by the bacterium *Bordetella pertussis*. It causes ribosylation of the Gαi-subunit, on a cysteine in the α5-helix (see Figure 3-7), thereby, preventing its interaction with G-protein-coupled receptors and thus preventing its activation. By removing an inhibitory constraint, the cells infected by the toxin produce more cAMP. Members of the Gαi family of G-proteins are all sensitive to PTX (GNAT1–3, GNAO1, GNAI1–3). The clinical manifestation is "whooping cough," a consequence of toxin-infected motor neurons that control cough reflexes.

Cholera toxin (CTX) is a virulence factor secreted by the bacterium *Vibrio cholerae*. It causes ribosylation of the Gαs subunit on an arginine near the nucleotide-binding pocket (R178 as shown in Chapter 2, Figure 2-30), thereby, preventing its capacity to hydrolyze GTP. The G-protein remains active and this leads to an enhanced stimulation of adenylyl cyclase, hence an increase in cAMP. The Gα-subunits GNAS1, SNAO1, GNAT1, -2, and GNAZ are sensitive to CTX. The clinical manifestation is diarrhea, a consequence of inappropriate control of ion transport across the gut epithelium. For G-protein names, see Chapter 2, Figure 2-29.

A big family of modular enzymes across all animal kingdoms

Until now we have dealt with human adenylyl cyclase, belonging to class III, but across all kingdoms, with the possible exclusion of plants, we recognize six different classes. They are particularly divergent in their domain composition which may reflect the great variety of primary signals that act through the production of cAMP ((pher)hormones, neurotransmitters, nutrients, ion-composition, or bicarbonate). Class I adenylyl cyclases are present in many Gram-negative bacteria. In *E. coli*, cAMP levels inversely mirror the presence of glucose outside the bacterium (see Chapter 2, Figure 2-39). cAMP serves as a ligand for transcription factors that control expression of operons like the lac operon and it enables the bacterium to shift from one source of sugar to another (in this particular case from glucose to lactose). Class II adenylyl cyclases are soluble and are present in certain pathogens such as *Bacillus anthracis* or *Bordetella pertussis*. Members of this class serve as extracellular toxins (see textbox cholera and pertussis toxin) that penetrate into mammalian cells and generate cAMP (upon binding of Ca^{2+}/calmodulin from the host). It is not clear what role they play inside the bacteria self. Most adenylyl cyclases belong to Class III and they are found in both prokaryotes and eukaryotes. In bacteria, elucidation of structure, regulation, and function of class III adenylyl cyclases is just at the beginning. In general, it appears that bacterial class



genename	distribution	Gαs	$G\alpha_i$	Gβγ	Ca^{2+}/ calmod	PKA	PKC	Ca^{2+}
ADCY10	epidimydus >other tissues							+
ADCY9	widely distributed	+	-					
ADCY7	widely distributed	+		+			+	
ADCY4	widely distributed	+		+				
ADCY2	brain, lung	+		+			+	
ADCY3	predominantly olfactory	+			+		+	
ADCY8	neural tissues	+			+			
ADCY1	neural tissues	+	-	-	+			
ADCY5	heart>brain	+	-			-	+	-
ADCY6	heart, brain>other tissues	+	-			-	-	-

FIGURE 3-13 Multiple regulation mechanisms of adenylyl cyclase. (a) Binding of Gαs to C2a results in stimulation of the enzyme, whereas binding of Gαi to a similar binding site on C1a leads to inhibition. Gβγ subunits and Ca^{2+}/calmodulin bind C2a and cause stimulation of the enzyme. Protein kinase C phosphorylates C2a and this too causes activation. Elevated levels of Ca^{2+} compete with metal ions (Mg^{2+} or Mn^{2+}) for the members ADCY5 and -6 and this hinders activity, whereas it stimulates in the case of ADCY10. (b) Adenylyl cyclase constitutes a family of 10 members, which, with the exception of ADCY10, are transmembrane proteins principally regulated by G-proteins. Note that regulation mechanism varies from one member to another. The list is not exhaustive because we have omitted inconsistent experimental findings for regulation by Gβγ, Ca^{2+}/calmodulin, and PKC.

III adenylyl cyclases are individually shaped to specific needs in processes such as osmoregulation, chemotaxis, phototaxis, or pH regulation. The role of type III adenylyl cyclase is most thoroughly studied in *Dictyostelium* where cAMP serves as an intracellular messenger but can also be secreted during starvation. It then serves as a relay signal that initiates and coordinates amebal aggregation into slug-like structures which eventually form spores in order to survive the lack of nutrition (Kay et al., 1999). In ciliates, such as *Paramecium* or *Tetrahymena*, the enzyme is composed of an ion-channel domain, with a canonical potassium-pore loop, fused to a single adenylyl cyclase domain. cAMP production is stimulated by potassium conductance that originates from changes in the ion milieu. In yeast, extracellular glucose triggers cAMP formation via a Ras-regulated adenylyl cyclase. Ras is a monomeric G-protein. The rise in cAMP stimulates glycolysis, to make use of the abundance of glucose, and causes cell proliferation, because the conditions are favorable (Thevelein and de Winde, 1999). The class III adenylyl cyclases are good examples of modular enzymes with a catalytic domain linked to a variety of protein interaction- or other catalytic domains (Figure 3-14) (Gancedo, 2013). Classes IV–VI have only been assigned tentatively and they are represented by one or two species. Class IV is found in *Aeromonas hydrophila* and *Yersinia pestis*. Class V is identified in the anaerobic bacteria *Prevotella ruminicola* and class VI occurs in the CyaC isoenzyme from *Rhizobium etli* (Linder and Schultz, 2003).

cAMP-BINDING PROTEINS

Mammalian cells possess three types of cAMP-binding proteins: the regulatory subunits (RI or RII) of PKA, the Rap1 nucleotide-exchange protein EPAC, and cAMP-gated ion channels (HCN, CNG, EAG) (Figure 3-14(b)) (Gancedo, 2013). PKA plays a role in the regulation of metabolic enzymes, ion channels conductance, and transcription (return to Chapter 2, Figure 2-43). EPAC is a GTP-exchange factor that leads to activation of the monomeric GTPase Rap1 or -2. These GTPases play a role in cell polarity, excitation–contraction coupling in the heart, cell migration, and, in fine-tuning, the rate of insulin secretion (Gloerich and Bos, 2010). The cAMP-gated ion channels all carry a cyclic nucleotide-binding domain in their carboxy terminal region, which can be selective for cGMP, for cAMP, or for both. The channels show distinctive functional characteristics. The HCN channel, hyperpolarization-activated cyclic nucleotide-modulated channel, alters cardiac rhythmicity by letting Na^+ entering the cell (depolarization) in the diastolic phase of contraction, hence its name “pacemaker channel” (Larsson, 2010). The CNG, for cyclic nucleotide-gated channel, are prominent in sensory cells related to vision or olfaction. In photoreceptors,

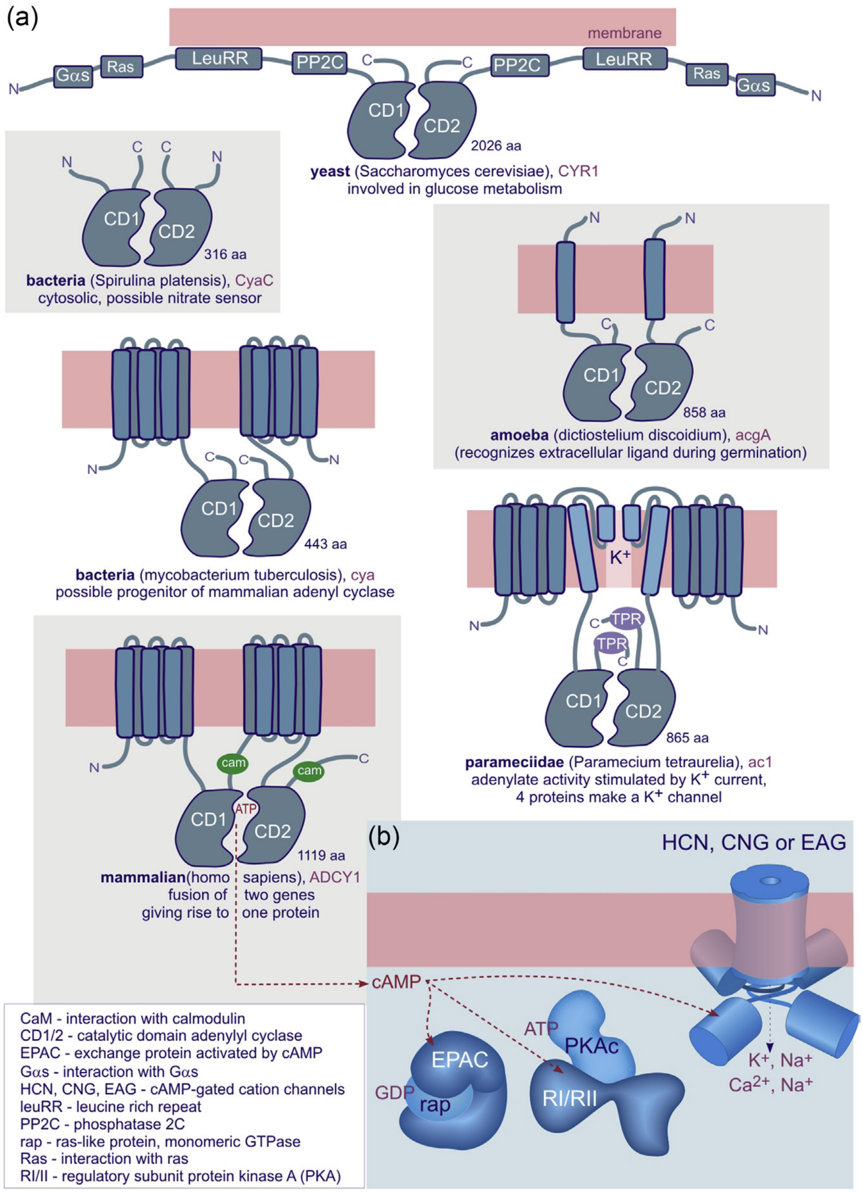

(a)
membrane
N
Gαs
Ras
LeuRR
PP2C
C
CD1
CD2
2026 aa
yeast (Saccharomyces cerevisiae), CYR1
involved in glucose metabolism
316 aa
bacteria (Spirulina platensis), CyaC
cytosolic, possible nitrate sensor
858 aa
amoeba (dictiostelium discoidium), acgA
(recognizes extracellular ligand during germination)
443 aa
bacteria (mycobacterium tuberculosis), cya
possible progenitor of mammalian adenyl cyclase
K+
TPR
865 aa
parameciidae (Paramecium tetraurelia), ac1
adenylate activity stimulated by K+ current,
4 proteins make a K+ channel
cam
ATP
1119 aa
mammalian(homo sapiens), ADCY1
fusion of two genes
giving rise to one protein
(b)
HCN, CNG or EAG
cAMP
EPAC
GDP
rap
ATP
PKAc
RI/RII
K+, Na+
Ca2+, Na+
CaM - interaction with calmodulin
CD1/2 - catalytic domain adenylyl cyclase
EPAC - exchange protein activated by cAMP
Gαs - interaction with Gαs
HCN, CNG, EAG - cAMP-gated cation channels
leuRR - leucine rich repeat
PP2C - phosphatase 2C
rap - ras-like protein, monomeric GTPase
Ras - interaction with ras
RI/II - regulatory subunit protein kinase A (PKA)

FIGURE 3-14 Schematic models of domain composition of some class III adenylyl cyclases and the targets of cAMP in mammalian cells. (a) The domain composition of adenylyl cyclase across different species nicely illustrates the modular approach of protein composition. Addition of sensor modules, transmembrane helices, or leucine-rich regions couples the enzyme to different input signals and determines subcellular localization. In the case of the mammalian gene, two adenylyl cyclase genes have been fused into one. *(Image adapted from Gancedo (2013).)* (b) cAMP regulates activity of three types of target proteins. One is a nucleotide-exchange protein, EPAC, which activates the monomeric GTPase rap, the second is the regulatory subunit (RI/II) of PKA, and the third is a small family of cAMP-gated cation channels (HCN, CNG, EAG).

they respond to cGMP which in olfactory sensory neurons are sensitive to both cAMP and cGMP. They too regulate the frequency of action potentials by partial depolarization of the membrane (influx of Ca^{2+} and Na^{+}) (Craven and Zagotta, 2006). Finally, EAG, for ether-a-go-go, are also cation channels that were discovered because of ether-induced leg-shaking in *Drosophila* mutants. An EAG-related gene, which contains both EAG and HCN domain characteristics, plays a role in epilepsy, schizophrenia, cancer, and its mutations lie at the basis of the cardiac long-QT syndrome, a cardiac depolarization disorder that predisposes to arrhythmia. We return in more detail to some of these effectors in a later part of the chapter.

PHOSPHOLIPASE C

The phospholipase family

Phospholipids are components of cellular membranes and they come in a large variety. They play an essential role in separating the outside from the inside of the cell but they also intervene in signaling processes after being modified by phospholipases. Phospholipids possessing a glycerol backbone contain four ester bonds, all of which are potentially susceptible to enzyme-catalyzed hydrolysis (Figure 3-15(a)). Hydrolysis at three of these positions gives rise to products that are either second messengers or substrates for enzymes that yield further signaling molecules.

Phospholipase A_2 (PLA_2) hydrolyzes mostly phosphatidylcholine yielding a lysophospholipid and releasing the fatty acid bound to the 2-position, generally arachidonate. Arachidonate is the substrate for the formation of prostaglandins and leukotrienes (potent signaling molecules with autocrine and paracrine effects involved in pathological processes such as inflammation). Phospholipase D cleaves phosphatidylcholine (the major membrane phospholipid) to form phosphatidate and water-soluble choline. Removal of the phosphate by phosphatidate phosphohydrolase produces diacylglycerol (DAG, a second messenger).

PLC removes the inositol ring from phosphoinositol 4,5-bisphosphate (PIP_2) thus creating two second messengers; one cytosolic, inositol 3,4, 5-trisphosphate (IP_3) and the other membrane-bound, DAG (Figure 3-15(b)). It should be noted that in addition to the PLC, PI-4,5-P_2 is also the substrate of the phosphoinositide 3-kinases (PI 3-K) that cause further phosphorylation, generating the lipid second messenger PI-3,4,5-P_3 (we return to this lipid kinase in Chapter X, “regulation of food intake”).

The first hints of a role for the inositol-containing phospholipids in cell regulation emerged in 1953 (Hokin and Hokin, 1953). Twenty years elapsed before a viable proposal concerning their role was forthcoming. Michell (1975) then pointed to the striking correlation between the activation of receptors that cause an increase in the metabolism of phosphatidylinositol

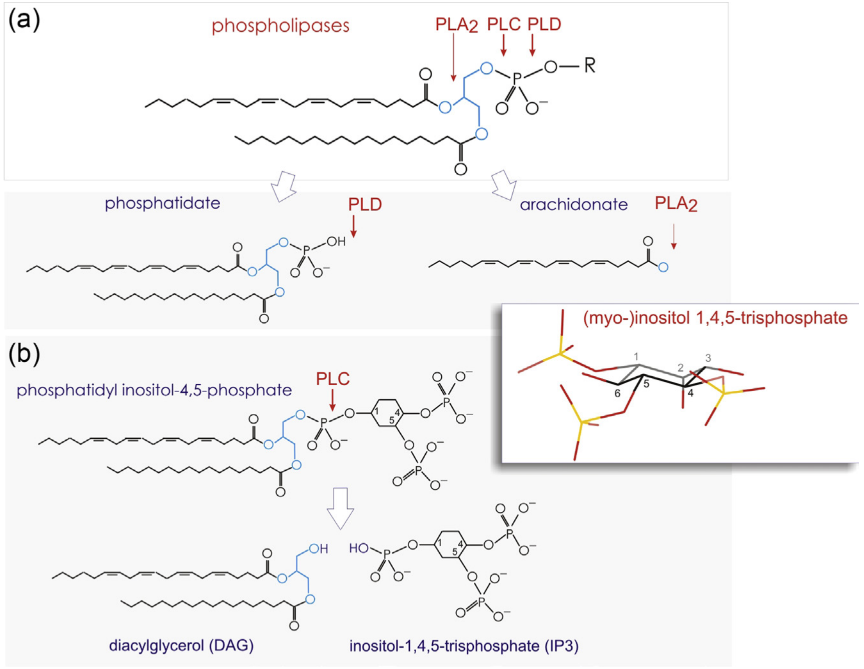

(a)
phospholipases
PLA2 PLC PLD
O—R
phosphatidate
PLD
OH
arachidonate
PLA2
(b)
phosphatidyl inositol-4,5-phosphate
PLC
(myo-)inositol 1,4,5-trisphosphate
1
2
3
4
5
6
HO
OH
diacylglycerol (DAG)
inositol-1,4,5-trisphosphate (IP3)

FIGURE 3-15 Phospholipids, phospholipases, and the production of second messengers. (a) Phosphatidate and choline are the products of phospholipase D (PLD) of which the main substrate is phosphatidylcholine. Arachidonate, together with a lysophospholipid, is the product of phospholipase A2 (PLA_2). (b) Composition of phosphatidylinositol 4,5-bisphosphate (specifically, 1-stearoyl, 2-arachidonoyl PI-4,5-P_2) and its breakdown products after intervention of phospholipase C (PLC): inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The glycerol backbone is blue. The biological form of inositol, based on cyclohexane, is myo-inositol, in which the hydroxy substitutions at positions 1, 3, 4, 5, and 6 are all equatorial, while the 2 position is substituted axially (insert on the right).

(PI) and activation of processes such as secretion and the contraction of smooth muscle that are dependent on Ca^{2+}. Although a PLC-catalyzed reaction was inferred, the precise identities of the substrate and the resulting second messenger(s) remained elusive. Then it was found that the rate of depletion of the poly-phosphoinositides, in particular, phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) (Figure 3-15) and not phosphatidylinositol itself, correlates with the onset of cellular activation (Creba et al., 1983). The water-soluble product IP_3 resulting from the hydrolysis of PI-4,5-P_2, was found to release Ca^{2+} from intracellular storage sites when introduced into permeabilized pancreatic cells (Streb et al., 1983). Within a very short time, it was shown that this mechanism is ubiquitous and that the immediately accessible Ca^{2+} stores are present in the endoplasmic reticulum which is endowed with receptors for IP_3. The hydrophobic product of PI-4,5-P2 breakdown is DAG and this is retained in the membrane bilayer. It causes the activation of protein kinase C (developed in Chapter 9) (Nishizuka, 1992).

The catalytic domain is a TIM barrel

PLC is a multidomain protein of which the catalytic site constitutes a TIM barrel, so named after the first enzyme discovered with a similar fold, triosephosphate isomerase. The barrel is composed of an inner lining of eight β-sheets, containing the catalytic residues, surrounded by an outer lining of eight α-helices (referred to as the eightfold αβ motif or $(\beta\alpha)_8$). The barrel is an assembly of two boxes, the X- and Y-box, each contributing 4α′s and 4β′s. The two boxes are connected by a linker of variable length (Figure 3-16). The TIM barrel is one of the ancient folds with considerable sequence diversity because the base of the β-sheets can easily be reengineered to bring diverse combinations of residues into contact, thereby, creating novel catalytic environments (Grant et al., 2004). Proteins carrying this barrel structure are nearly always enzymes involved in cell metabolism (sugar isomerase, pyruvate kinase, transglycosylase, or nuclease) with the exception of PLC which is involved in signaling. Whereas many enzymes use metal ions, PLC requires Ca^{2+} for both coordination of the inositol ring and catalysis (hydrolysis of the phosphoester bond). This function should not be confused with the membrane-recruitment role of Ca^{2+} (discussed below).

With respect to the regulation of activity, activators seem not to induce general conformational changes in the catalytic domain, but rather, recruit and optimally orient the enzyme at the plasma membrane (or the nuclear envelope) to overcome electrostatic repulsion and cause displacement of the linker that connects the X- and Y-box. The different domains surrounding the TIM barrel facilitate, with the appropriate signal, the recruitment at the membrane (Figure 3-17).

The isoenzymes of PLC and their mode of regulation

Thirteen functional mammalian isoforms of PLC have been recognized. These fall into six classes, β, γ, δ, ε, η, and ζ, based on protein domain composition (Figure 2-18). In addition, two inactive variants have been identified, the so-called PLC-like enzymes, PLC-L. Before we provide a brief description of the domain composition of the different isozymes, it is important to understand that these domains, somehow, have a role in positioning the TIM barrel near its substrate, in other words, have a role in firmly attaching the enzyme to the membrane. Recruitment occurs through binding to membrane-bound G-proteins (various domains), through Ca^{2+}-mediated insertion of the C2 domain into the membrane or through attachment of the SH2-domain to tyrosine-phosphorylated membrane proteins. Besides

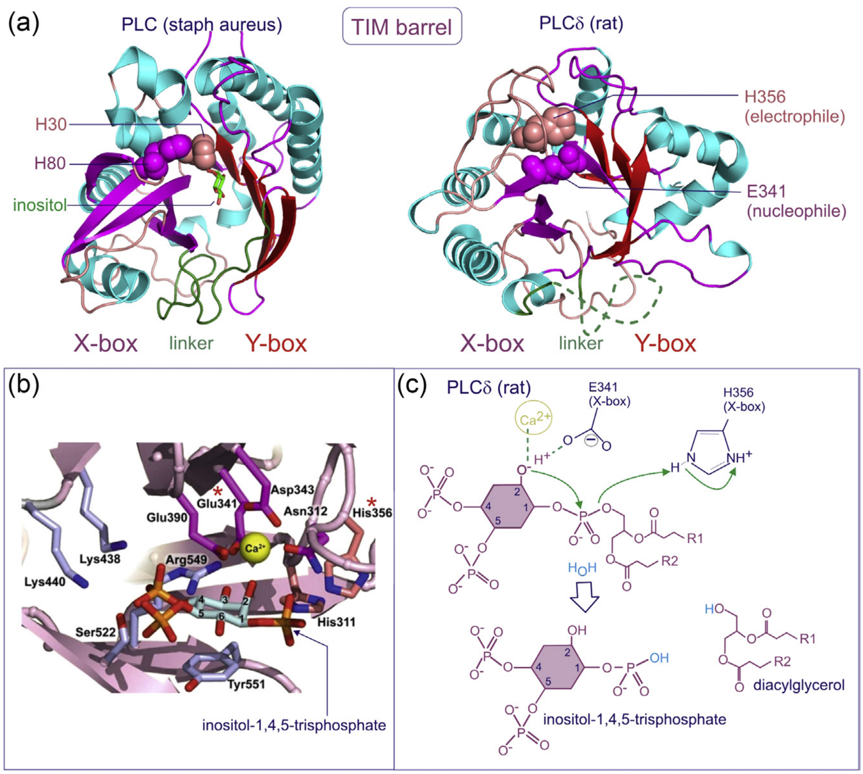


FIGURE 3-16 TIM barrel of bacterial and mammalian PLC and its mode of action. (a) The catalytic domain of PLC is a TIM barrel, named after the enzyme where this configuration was shown for the first time, triosephosphate isomerase. It is a cup-like structure of which the inner lining is composed of eight β-strands and the outer layer of eight α-helixes. The domain is comprised of two halves, an X- and Y-box (cyan/pink and cyan/red respectively), each contributing four β-strands and four α-helices. Essential catalytic residues are highlighted as spheres, for the rat enzyme E341 and H356, both located in the X-box. pdb: 3v1h, 2isd. (b) An extensive network of salt-bridge and hydrogen-bond interactions is formed between inositol phosphate and Asp312, Glu341, Asp343, and Glu390. These also coordinate a Ca^{2+}-ion which renders the 2-hydroxyl more nucleophile (by pushing away the H^+). *(Image adapted from Gresset et al. (2012).)* (c) Proposed catalytic mechanism. Hydrolysis of phosphatidylinositol 4,5-bisphosphate follows a general acid (histidine356)/base (glu341) catalytic scheme. Although not shown in the figure, the reaction occurs through the intermediate of a cyclic structure, where phosphate is associated with the 1- and 2-hydroxyl group of inositol. This bond is broken by water.

the catalytic domain, all isozymes contain EF-hands and a C2-domain, both of which are originally qualified as Ca^{2+}-binding domains but not all are functional as such. The C2-domain was first discovered in protein kinase C, where it serves to attach the kinase to the membrane in the presence of elevated (>150 nM) concentrations of intracellular free Ca^{2+}. Despite their

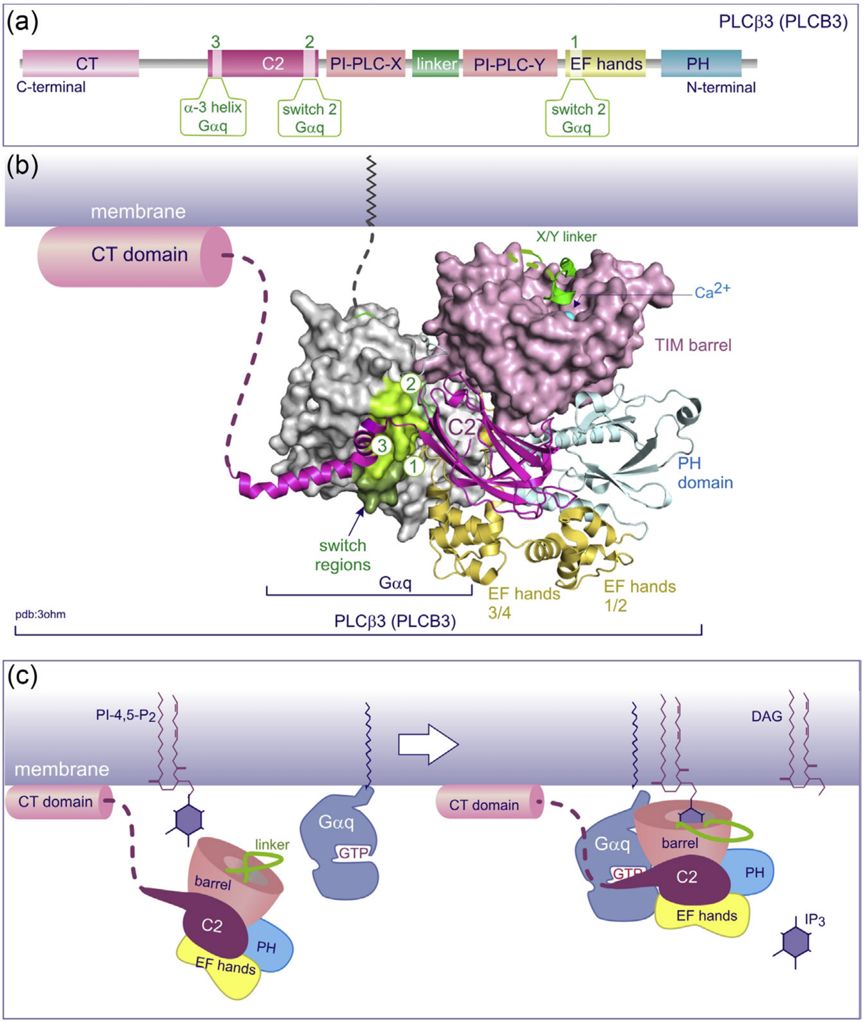


FIGURE 3-17 Structural organization of PLCB3 and proposed mode of activation of phospholipase C. (a) Domain architecture of PLCB3 and interaction sites with Gαq. The same color scheme has been employed in the surface/cartoon representation of the enzyme below. The numbers 1, 2, and 3 (in green) denote the contacts between enzyme and the Gαq-subunit. Note that, in order to match the domains with the relative position in the structure, we have oriented the C-terminal at the left (the amino acid sequence starts from the right). (b) Structure of PLCB3 bound to Gαq. The G-protein α-subunit (gray) and the catalytic TIM barrel (pink) are shown in surface representation. The switch regions are green. The complex has been slightly tilted to show the Ca^{2+}-ion (in blue) which represents the catalytic site of the enzyme and the linker peptide, which precludes access of substrate and must be replaced for activation (mechanism not known). The pleckstrin homology (PH)-domain, edema factor (EF)-hands, and C2 domain, shown in cartoon representation, contour the catalytic barrel and, depending on the isozyme, play a role in recruitment to the membrane. pdb:3ohm (c) Proposed model of PLCB3 activation by Gαq. Loading of the heterotrimeric G-protein with GTP causes conformational changes in the switch regions and in the case of Gαq this leads to binding of the PLC β-isozymes. The ensuing recruitment at the plasma membrane, the optimal orientation of the enzyme, and the displacement of the linker region constitute the activation process. Alternative recruitment modes, for other PLC isozymes, are Ca^{2+}-mediated insertion of C2 into the membrane, attachment of the SH2 domain (not shown) to a tyrosine-phosphorylated transmembrane protein, or binding to membrane-bound monomeric G-proteins (Rap1, Ras, Rac, RhoA) and Gβγ subunits. The relative position of the EF-hands, PH- and C2-domain may vary between different isozymes because of flexible linker regions.

presence, only members of the δ- and the ζ-isozymes are regulated by Ca^{2+}. The majority of isozymes carry a pleckstrin homology domain (PH-domain) originally qualified as domain that binds phosphatidylinositol lipids within biological membranes. In the case of γ, η, and δ, the domain serves indeed to facilitate membrane attachment but in the case of the β-isozymes, the role of the PH-domain is taken by the C-terminal (CT) domain. In the case of PLCB2, the PH-domain serves to bind the monomeric G-protein Rac. PLCE1 and PLCG1/2 are exceptional with respect to their domain composition. PLCE1 has inserted a Ras-association domain which binds H-ras or Rap1 (both monomeric G-proteins) and causes membrane recruitment and activation of the phospholipase. It also carries a nucleotide exchange (GEF) domain which is able to activate Rap1 (and perhaps Ras). As a consequence, the activated enzyme will not only hydrolyze PIP_2 but it will also cause further activation of the monomeric G-proteins (a feed-forward loop). Lastly, PLCG1/2 have inserted three Src-homology (SH) domains, SH2 and SH3, in their linker region (connecting the X- and Y-box) and this has made the enzyme sensitive to phosphotyrosine-containing sequences. The SH2 domain was discovered as region in Src that binds a phosphotyrosine in the context of three specific amino acids. Growth factors and antigens bound to T cells signal through tyrosine protein kinases and indeed the γ-isozymes are recruited to the membrane upon activation of these receptors (Gresset et al., 2012). Abbreviations are explained in Figure 3-18.

PLC-β has a GTPase-activating function

Members of the β-isozymes are recruited by α-subunits of the heterotrimeric G-proteins of the Gq subclass (Gαq, Gα11, Gα14, and Gα15). This interaction only occurs when the Gα-subunit is loaded with GTP and involves on the one side the switch-2 region and an adjacent α3-helix and the other the EF-hands and the C2-domain of the phospholipase (see Figure 3-17(b) and 3-18(b)) (Waldo et al., 2010). Interaction with the EF-hands also facilitates the intrinsic GTP-ase activity leading to hydrolysis of GTP into GDP and Pi (i.e., rendering the G-protein inactive). This GTPase-activating effect (GAP function) was first identified for PLCB1 (Berstein et al., 1992) but later be shown to apply for all β-isoforms (Ross, 2008). This activity assures a rapid turnoff of Gαq-stimulated signaling once the receptor is dissociated from its ligand and would suggest that it serves to reduce the extent of the signal. Paradoxically, PLC increases the time in which Gq is in its GTP-bound state. This occurs because the increased rate of inactivation is accompanied by an equally increased rate of re-activation, and the net result is an increase in the magnitude of the intracellular signal. For this to occur, Gαq has to remain associated with a ligand-bound receptor so that the GDP is immediately replaced by GTP (the GEF function of the seven-membrane spanning receptors). Indeed, it is proposed that the presence of PLCβ isozymes promotes

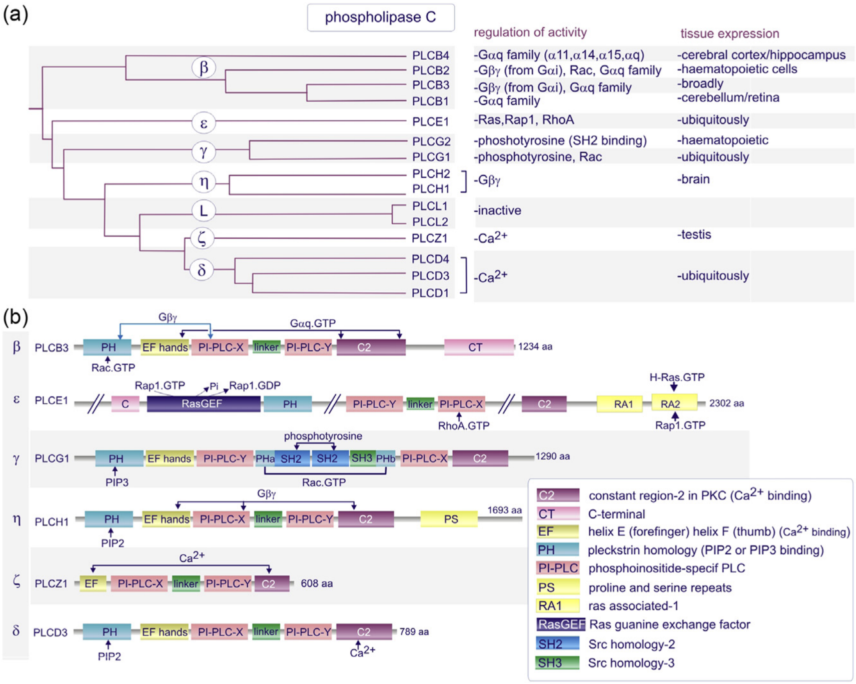


FIGURE 3-18 Phylogenetic tree and domain architecture of the phospholipase C (PLC) enzymes: (a) Phylogenetic tree (evolutionary relationship) of members of the PLC family. (b) Domain architecture of the different members of the PLC family. The common core of the isozymes includes a pleckstrin homology (PH)-domain, a series of EF-hands, a catalytic TIM barrel, with an X- and Y-box, and a C2 domain. Members of the β, ε, and η isozymes have additional domains that make the protein much longer. The arrows indicate interaction sites with G-proteins (Rac, Rap1, H-Ras, Gαq, and Gβγ), with phosphoinositide lipids (PIP$_2$, PIP$_3$), and with Ca^{2+} (different binding site from the catalytic TIM barrel). PLCE1 contains a second catalytic domain which is a GTPase-activating protein domain (GEF) for the monomeric G-protein Rap1 (and perhaps Ras). The insertion of SH2 domains, in between the X- and Y-box, renders the γ-isozymes responsive to receptors that signal through tyrosine protein kinases (such as growth factor receptors and antigen receptors in T cells). PLCL1 and -2 are not shown, these members are catalytically inactive.

G-protein/receptor interaction, a phenomenon referred to as "kinetic scaffolding" (Ross, 2008). The same mechanism independently evolved for a large number of proteins that interact with heterotrimeric G-proteins such as the regulator of G-protein signaling (RGS) proteins (Tesmer et al., 1997a) adenylyl cyclase and RhoGEF (see Chapter 2 "An Introduction to Signal Transduction," section "GEFs and GAPs").

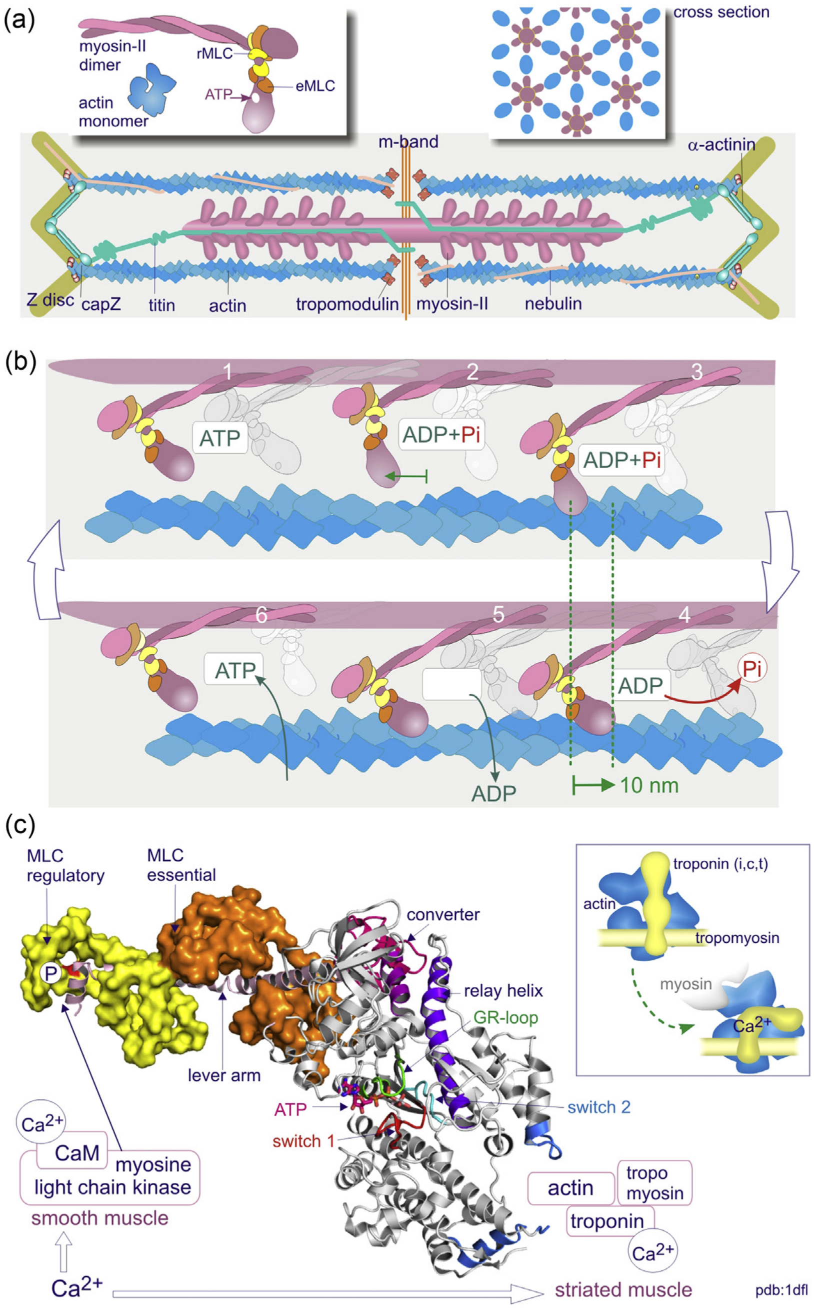
Having dealt extensively with the characteristics of receptors and effectors that intervene in the signaling events of adrenaline and noradrenaline, we next place them in the context of muscle contraction and relaxation.

MUSCLE CONTRACTION: STRIATED VERSUS SMOOTH MUSCLE

Muscle contraction constitutes the sliding of opposing actin filaments over myosin-motor filaments, first clearly demonstrated around 1954 with the help of high-resolution phase contrast- and electron microscopy (Figure 3-19) (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). The motor protein myosin has characteristics of a protein kinase and of a G-protein because its ATP-binding pocket both includes a glycine-rich (GR) loop (or P-loop), like in protein kinases (return to Chapter 2, Figure 2-35) and a set of switch regions, similar to G-proteins (return to Chapter 2, Figure 2-25). The chemical cycle underlying the mechanical cycle of the myosin-head resembles that of a GTPase; where myosin is charged with ATP, followed by hydrolysis into ADP + Pi and a subsequent loss of Pi and finally a loss of ADP. The conformational changes in the switch regions are in the order of 10 angstroms but are amplified by the "converter" and "lever arm" of myosin and result in a movement of the head of nearly 10 nM (the power stroke) (Rayment et al., 1993; Kull and Endow, 2013). Like for GTP-binding proteins, the cycle occurs spontaneously, but is dramatically enhanced, a near 1000-fold, during muscle labor. However, unlike GTP-binding proteins, the conformational change of the switch regions does not occur immediately after hydrolysis of ATP, because both ADP and Pi remain trapped in the protein. It requires the subsequent

FIGURE 3-19 Organization of contractile units, the cross-bridge cycle and structure of myosin-head. (a) Striated muscles have highly organized contractile units which are named sarcomeres. They contain opposing actin (thin) and a central myosin (thick) filament. The actin filaments are stabilized by the cap-proteins tropomodulin and capZ, and their attachment to the Z-discs involves α-actinin and nebulin. Nebulin also acts as a ruler, to regulate actin filament length (1.0–1.3 µm). Myosin is held at the center of the sarcomere, in order to keep contact with opposing actin filaments, through the elastic protein titin (a "Titan-size" protein of 34,350 amino acids). For clarity reasons, only a single copy of nebulin and titin are shown at either side of the sarcomere. (b) Brief description of the cross-bridge cycle; (1) myosin charged with ATP, (2) hydrolysis of ATP and reversal of the power stroke, (3) myosin charged with ADP + Pi has high affinity for actin and (4) actin binding leads to changes in the switch regions which in first instance causes a loss of Pi, followed by a change in the position of the head relative to the arm (power stroke). The actin filament slides at a distance of roughly 10 nM. (5) Subsequent changes in the switch region allow the escape of ADP, and (6) myosin is recharged with ATP, causing the detachment from actin. A new cycle commences. In striated muscle, the events are arrested by blockage of interaction between myosin and actin, in smooth muscle by blockage of the head movement by the regulatory MLC (mechanism not yet elucidated). (c) The structure of the myosin-head illustrates the central position that the ATP-binding site takes in the cross-bridge cycle; with on the left, the converter and lever arm and on the right, the actin-binding site. The lever arm is enwrapped by two myosin light chains, named essential (orange) and regulatory (yellow) MLC. Intracellular free Ca^{2+} plays an essential role in the control of the cross-bridge cycle. In striated muscle, it frees the myosin-binding site on actin by binding to troponin and causing a slight displacement of the troponin/tropomyosin complex. In smooth muscle, Ca^{2+} activates myosin light-chain kinase, leading to phosphorylation of regulatory MLC and, as a consequence, relief of the blockage of the cross-bridge cycle.

▶


(a)
myosin-II dimer
rMLC
eMLC
ATP
actin monomer
cross section
m-band
α-actinin
Z disc
capZ
titin
actin
tropomodulin
myosin-II
nebulin
(b)
1
2
3
ATP
ADP+Pi
ADP+Pi
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5
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ATP
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converter
relay helix
GR-loop
lever arm
ATP
switch 1
switch 2
troponin (i,c,t)
actin
tropomyosin
myosin
Ca2+
Ca2+
CaM
myosine light chain kinase
smooth muscle
Ca2+
actin
tropo myosin
troponin
Ca2+
striated muscle
pdb:1dfl

binding to actin (which occurs because of increased affinity) that leads to changes in the switch regions, which then causes Pi to escape and this provokes the power stroke. The loss of ADP follows immediately. Recharging the myosin with ATP weakens the interaction with actin and only when the two are fully detached, and the new ATP is again hydrolyzed, does the myosin head return to its original position (Goody, 2003).

A major difference between smooth and striated muscle, apart from the anatomical organization, resides in the control of the above described cross-bridge cycle. In the case of smooth muscle control is exerted on the movement of the head, which apparently is prevented by the regulatory myosin light chain, whereas in striated muscle control is exerted at the level of contact with actin. In both cases, Ca^{2+} plays a crucial role. In smooth muscle it controls the activity of myosin light-chain kinase (gene name MYLK, also known as MLCK) thereby, causing the phosphorylation of the regulatory light chain and allowing movement of the myosin-head, whereas in striated muscle it controls the displacement of troponin/tropomyosin, thereby, allowing contact between actin and myosin. The first is said to be slow, a matter of seconds, the second is fast, a matter of milliseconds (Figure 3-19(c)).

Andrew F Huxley, grandson of Thomas Henry Huxley (see Chapter 1, “Prologue,” section “Irritability, a Vital Phenomenon”), made an important contribution to the discovery of the sliding filaments in striated muscle but he shared the 1963 Nobel Prize in Physiology or Medicine with John Eccles and Alan Hodgkin “for his discoveries concerning the ionic mechanism involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane.” He, together with Alan Hodgkin, employed for the first time microelectrodes, which were inserted in the giant nerve fiber of squids, in order to study the flow of ions during the polarization and depolarization phase of the action potential. His pioneering work has helped to develop the chemical transmission theory of nerve transmission at synapses and at the neuromuscular junction. A second founder of muscle motor protein field is Hugh E Huxley, same family name but not related.

With respect to anatomical organization, all muscles employ organized actin/myosin contractile units. In smooth muscle, they are distributed rather haphazardly, whereas in striated muscle they are perfectly aligned in the form of sarcomeres (hence the striated appearance) (Figure 3-20). Whereas skeletal muscle is composed of huge fibers made of hundreds of fused muscle cells (myocytes), cardiac muscle is composed of individual

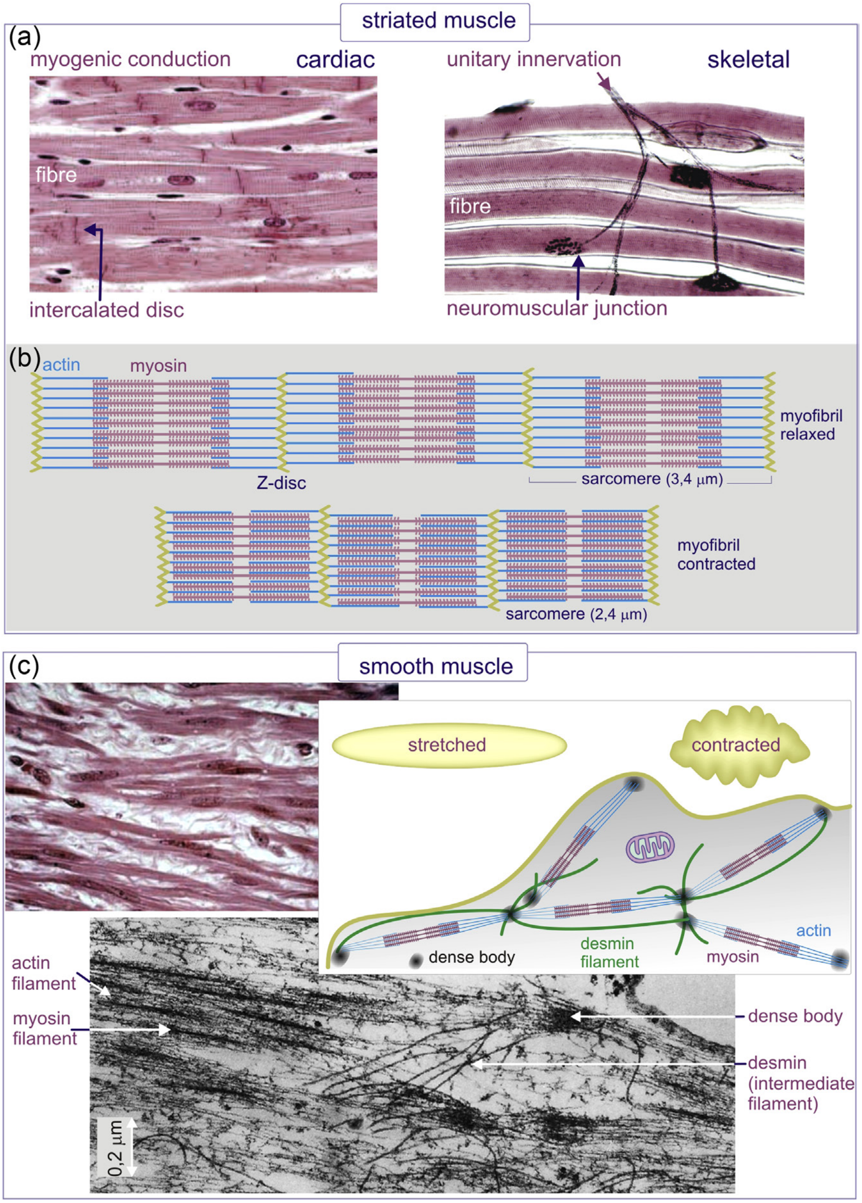

FIGURE 3-20 Histology of striated (cardiac and skeletal) and smooth muscle. (a) Muscle fibers of the heart are composed of individual cells attached through intercalated disks. These contain gap junctions that allow the conduction of action potentials (myogenic conduction). Muscle fibers are a fusion product of hundreds of myocytes. Multiple muscle fibers form a motor unit which is innervated by branches of a single motor neuron (unitary innervation). (b) Muscle fibers are composed of myofibrils and these are a repetition of numerous contractile units named sarcomeres. These are composed of opposing actin filaments (thin) that surround myosin filaments (thick). Actin is fixed to Z-disks and these constitute the boundaries of the sarcomere. Contraction comprises the shortening of the sarcomere, reducing its length from roughly 3.4 to 2.4 µm (a 30% reduction). (c) Smooth muscles are composed of individual cells, spindle-like, which harbor contractile units that resemble sarcomeres but are much less organized. Rather than Z-disks, the contractile units are connected by dense bodies and intermediate filaments (made of desmin). Electromicrograph of smooth muscle from Bond and Somlyo (1982).

cells, sometimes binuclear, connected through intercalated disks that contain large fields of gap junctions. Smooth muscle cells are also individual cells but depending on the type of tissue they either are connected by gap junctions (smooth muscle in arteries) or not (iris of the eye). Skeletal muscle functions in motor units, each with unitary innervation, giving rise to synchronized contractions initiated by action potentials (ionotropic regulation, nicotinic receptor) at multiple neuromuscular junctions. Cardiac contraction is also initiated by action potentials but these are generated by autorhythmic cells of the sinoatrial node (SA node, the dominant pacemaker), and conduction occurs in a progressive wave that diffuses through the tissue from cell to cell (myogenic conduction). The diffusion occurs through gap junctions in the intercalated discs. Cardiac muscle is nevertheless innervated, by nerve terminals of both the sympathetic (catecholamines) and parasympathetic system (acetylcholine). This innervation serves to modulate both rhythmicity and contractile force (metabotropic regulation by adrenergic and muscarinic receptors). Depending on the tissue, smooth muscle contraction is either initiated by action potentials, as occurs in the gut by autorhythmic cells, or by the production of second messengers (metabotropic) as occurs in the arteries and the trachea. Finally, innervation of smooth muscle varies from unitary, occurring in the gut, and multiunit innervation, occurring in the ciliary muscle of the eye. Nerves in smooth muscle and cardiac tissue do not make elaborate neuromuscular junctions, instead neurotransmitter is released from varicosities from where the neurotransmitter diffuses into the tissue (Figure 3-28).

CONTRACTION WAVES IN THE HEART

The action potential spontaneously generated in the SA node travels across the membrane of atrial myocytes, then passes through the AV node and enters the ventricle through a rapid conduction via the His bundle and Purkinje fibers (modified myocytes with fast conduction of action potential). The action potential is propagated by voltage-sensitive Na^+-channels (SCN5A in the cardiac myocyte) situated in the plasma membrane (or plasmalemma). In the contractile tissue, it causes the opening of voltage-sensitive Ca^{2+} channels and this leads, in cooperation with the ryanodine receptor of the sarcoplasmic reticulum, to a Ca^{2+}-influx into the cytoplasm. The ensuing Ca^{2+}-mediated displacement of troponin starts the cross-bridge cycle between actin and myosin.

In order to improve the contact between membrane and the contractile machinery, the plasmalemma penetrates into the cell by the way of T-tubules. These are surrounded by the sarcoplasmic reticulum (SR). Both plasmalemma and T-tubules harbor the ingredients needed for regulation of contraction by noradrenaline/adrenaline, namely β1-adrenoceptors, adenylyl cyclase, and numerous types of ion channels (Nikolaev et al., 2010). Figure 3-21 shows a brief outline of pacemaker activity, action potential conduction in the heart, and how it reaches the contractile machinery.

Etymological note. *Sarco* is Greek for muscle and is equivalent to the Latin *myo*. The vocabulary used in anatomy and histology freely interchanges the Greek and Latin root. For example, we speak of myofibrils and how they are composed of sarcomeres that comprise myosin-II filaments. We speak of a myocyte (muscle cell), containing a sarcoplasm (cytoplasm) and a sarcoplasmic reticulum (smooth endoplasmic reticulum) and which is surrounded by a sarcolemma (plasmalemma or plasma membrane).

ADRENALINE AS A CARDIAC INO- AND CHRONOTROPE MESSENGER

When autonomic nervous input into the heart is blocked, the spontaneous depolarization rate of the SA node is 100 times per minute (on average) and as a consequence, the heart will beat at that rate (intrinsic rate). However, an average resting heart rate in an adult is around 70 beats per minute, and thus it follows that the heart is normally under inhibitory, parasympathetic, control ("vagal tone"). Increasing the heart rate, as occurs in anxiety or intense muscle labor, can be achieved in two ways. Initially by reducing parasympathetic control, the rate may go up as much as 100 beats, and then by an increase in sympathetic control through the release of noradrenaline from nerve terminals. Concomitantly, the sympathetic system will cause the release of adrenaline (and to a lesser extent noradrenaline) from the adrenal gland (return to Figure 3-2), providing an additional hormonal boost. As a result, we feel the heart pounding in our chest, beating both faster and stronger. Both neuronal and hormonal control reaches the heart at all levels, the nodes (SA and AV), the atria and ventricles, in a rather diffuse fashion.

Cardiac myocytes express at least six subtypes of adrenoceptors which include three β-receptors (ADRB1-3) and three α1-receptors (ADA1A, 1B, 1D). With respect to their expression levels, the β1-adrenoceptor (ADRB1) is the predominate one, it is four times higher expressed than β2 and many times more than β3 (Cannavo et al., 2013). β1-adrenoceptor, the focus of the next section, is expressed both on the cell crest (cell membrane) and in the T-tubules that penetrate the muscle fibrils and they signal from both sites (in contrast to β2-adrenoceptor, which is exclusively active in the T-tubules) (Nikolaev et al., 2010).

The β1-adrenoceptor is coupled to the heterotrimeric protein Gαs (GNAS1) and it causes the formation of cAMP by adenylyl cyclase (the

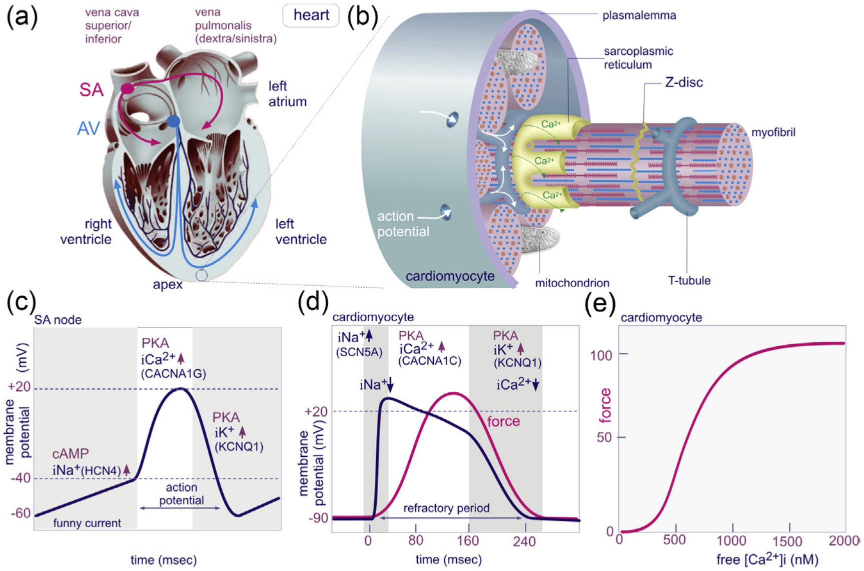

FIGURE 3-21 The heart and regulation of contraction waves. (a) The pacemaker of the heart, sinoatrial node (SA) is situated near the entry of the vena cava superior. The action potential spreads over the atria (left and right) and reaches the atrioventricular node (AV) where it is slowed down. It then enters the ventricles where the signal is rapidly conducted toward the apex and diffuses upward. The contraction wave, with the help of valves, pushes the blood flow from veins to arteries. (b) The cardiomyocyte is composed of multiple myofibrils, each of them being a concatenation of sarcomeres. The action potential is conducted inside the cardiomyocyte by invaginations (T-tubules) of the plasmalemma. These tubules contact the sarcoplasmic reticulum. The release of Ca^{2+} initiates the contraction phase. (c) Pacemaker activity is determined by three events. A slow inward Na^+ current (funny current or If) dictates the pacemaker potential (HCN4 channel predominant), once a threshold of −40 mV has been reached, Ca^{2+} channels open (CACNA1G) and start of action potential. When depolarization reaches +20 mV, K^+ channels open (KCNQ1) and the subsequent inward current brings the potential down to −60 mV. Noradrenaline and adrenaline affect all three events: cAMP increases conductivity of HCN4 (rapid rise in pacemaker potential), PKA-mediated phosphorylation increases the conductivity of the CACNA1G channel (more Ca^{2+} enters the cell) and PKA-mediated phosphorylation of KCNQ1 increases the outward K^+ current (rapid depolarization). At young age (below 20 years), the heart rate may go up from 90 (intrinsic rate) to 200 beats/minute). (d) The action potential generated by the SA or AV node reaches the contractile tissue and causes a rapid rise in Na^+ conductance (inward). Once the membrane is depolarized (+20 mV), the voltage-sensitive Na^+ channels shut and Ca^{2+} enters the cytoplasm (from the sarcoplasmic reticulum). The membrane repolarizes through an increased K^+ conductance (outward). The sequence can be repeated after 250 ms, meaning that the heart rate is limited to 240 beats/min. (e) Graph showing the relationship between intracellular free Ca^{2+} concentrations and contractile force. The more Ca^{2+} enters during the 240 ms, the stronger the heartbeat.

subtype ADCY5 is most active in the heart). Subsequent activation of PKA leads to phosphorylation of various target proteins that are involved in the regulation of Ca^{2+}, K^+, and Na^+currents in or out of the cytoplasm (Figure 3-22). With respect to the SA node, the net result is a shortened pacemaker potential (increased funny current) and a faster depolarization/repolarization, thereby, increasing rhythmicity (chronotropic effect). With respect to contractile tissue, the net result is increased intracellular free Ca^{2+}-transients which gives rise to more cross-bridges between actin and myosin and thus a stronger contractile force (inotropic action) (Bers, 2000). Noradrenaline and adrenaline also cause a more abrupt increase and subsequent decline in contractile force (which we consider a chronotropic effect), which increases pump efficacy at fast heart rates (Unglaub-Silverthorn, 2010).

ION CHANNELS, MEMBRANE POLARITY, AND INTRACELLULAR FREE CA^{2+}.

Ions are naturally charged and their movement across the membrane is determined by two factors: one, the membrane potential and two, the chemical gradient. The K^+ concentration is typically much higher inside the cell than outside, meaning that normally more ions will escape than return into the cell (stochastic process). However, the membrane potential of −90 mV opposes the K^+ outflow. For Na^+ the situation is reversed; the ion is enriched at the outside and the membrane potential facilitates its entry. As a result, when you open a cation channel which is permeable to Na^+ and K^+, initially only Na^+ will enter because K^+ is opposed by the membrane potential. The gradual depolarization will favor the outflow of K^+ and slow down the inflow of Na^+.

Increasing the conductivity of K^+ channels will cause a gradual hyperpolarization until it stabilizes at a new voltage were the two forces, "chemical and electrical" outbalance each other. Reducing K^+ conductivity will lead to a gradual depolarization until a new equilibrium is reached at a new higher membrane potential. In general, K^+ leakage is more important than Na^+ leakage and that explains why an equilibrium of flow (steady state or resting conditions) occurs at negative membrane potentials (inside relative to outside). Importantly, the Na^+/K^+-pump assures that the ion gradient remains firmly established (counteract the leakage).

Web resources

KEGG map 04261 http://www.genome.jp/kegg/kegg2.html.

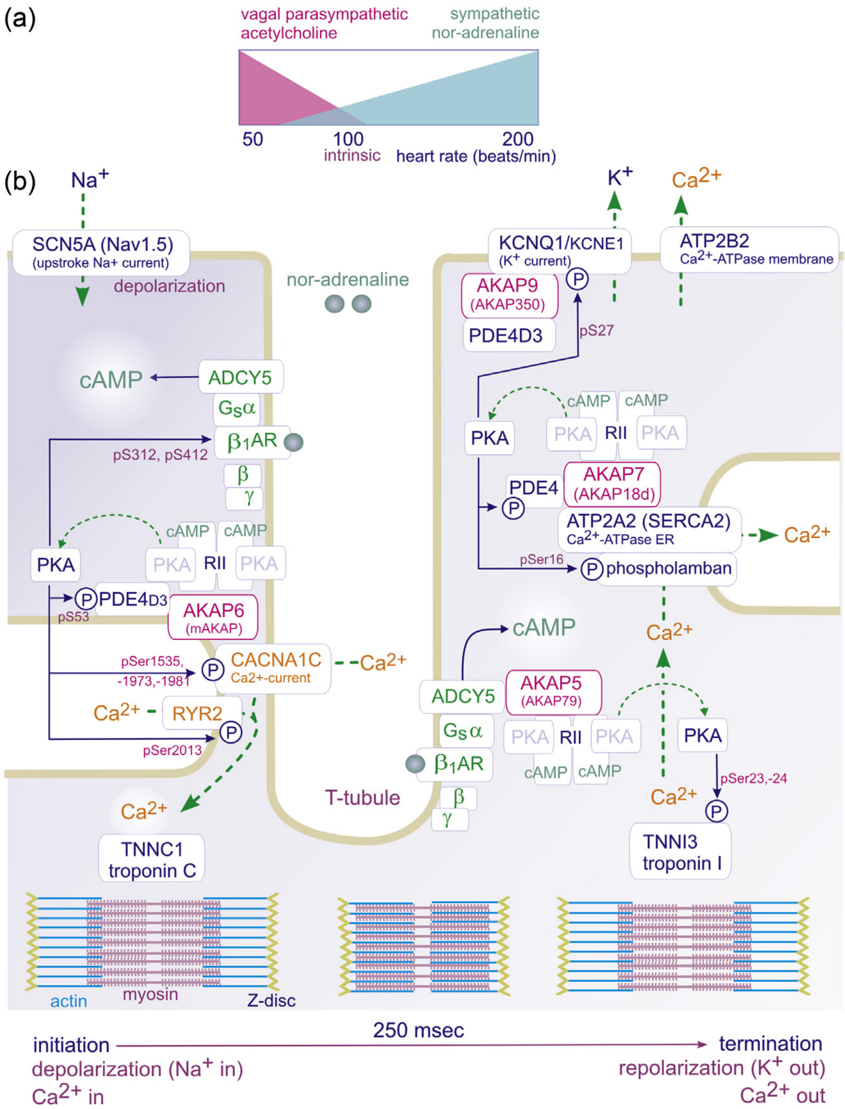

FIGURE 3-22 Schematic representation of events leading to enhanced cardiac contractility after addition of noradrenaline (or adrenaline). (a) The heart has an intrinsic contraction rate of roughly 100 beats per minute. Parasympathetic control (nervus vagus) reduces the rate and sympathetic control, noradrenaline, increases the rate (and force). (b) The inotropic (force) and chronotropic (rate) effect of adrenaline is caused by the production of the second messenger cAMP, as a consequence of the stimulation of adenylyl cyclase 5 (ADCY5) by Gαs. cAMP in turn interacts with the regulatory domain (RII) of protein kinase (PKA). Binding of cAMP to RII liberates and activates the PKA-catalytic domain. Substrates are two Ca^{2+}-channels, CACNA1C and RYR2, and their phosphorylation increases their conductivity. The consequence is an elevated influx of Ca^{2+} which gives rise to an increased number of

Activation of PKA

The holoenzyme PKA is a complex of two regulatory (gene name PRKAR) and two catalytic subunits (PRKAC). The heart expresses RI and RII (PRKAR1A or -B and PRKARIIA or -B). The regulators are associated with A-kinase-anchoring proteins (AKAPs) of different sorts and these play a role in the subcellular localization of the holoenzyme. Some anchoring proteins act as scaffold proteins, bringing together numerous regulators of signaling pathways in compact cassettes and it is generally proposed that this creates micro control-environments which dictate, in part, the specificity of cellular responses to agents that mediate their action via cAMP. There are 13 different human AKAPs, AKAP1–13, each with many alternative names and with specific interactions with PKA-regulatory subunits (type I or II or both) and numerous other proteins (Welch et al., 2010; Zhang et al., 2011; Fischmeister et al., 2006). The holoenzyme is normally inactive because the regulatory subunit hinders access of substrate to the catalytic cleft (return to Chapter 2, Figure 2-43). cAMP binds to the regulatory subunit and this brings about a conformational change which liberates PKA-C. The enzyme is now free to interact with substrate for as long as cAMP levels remain above a certain threshold. If the pulses of cAMP are short, only substrates in the immediate vicinity of the kinase will be reached, if the pulses are long, PKA-C may diffuse further into the cell (Figure 3-22).

Substrates of PKA in the cardiomyocytes

The substrates of PKA are numerous, but we will highlight those that provide a rough picture of the current understanding of how adrenaline and noradrenaline work. Starting from the action potential which enters

actin/myosin cross-bridges, hence an increase in force. Ca^{2+} binds the C-subunit (TNNC1) of troponin and this causes a conformational change that permits interaction between actin and myosin. PKA also phosphorylates phospholamban, a regulatory subunit of the Ca^{2+}-pump (ATP2A2) located in the membrane of the sarcoplasmic reticulum. Phosphorylation of phospholamban removes an inhibitory constraint and Ca^{2+} is taken up more effectively (chronotropic effect) and fully recharged Ca^{2+}-store will strengthen contractility in the subsequent beat (inotropic effect). PKA phosphorylates the troponin-i subunit (TNNI3) and this causes a reduced affinity for Ca^{2+} of the troponin complex. Through this action, Ca^{2+} more easily dissociates from the complex and, therefore, shortens the contraction phase (chronotropic effect). At the plasmalemma, PKA phosphorylates the KCNQ1 channel, giving rise to an increased outward K^+-current, a process that hastens repolarization of the membrane potential. Two phosphorylations by PKA serve as negative feedback loops. The first concerns phosphorylation and stimulation of phosphodiesterase-4 (PDE4). The enzyme converts cAMP into AMP (negative feedback) and this causes the return of PKA-C to its inhibitory subunit (RI/II). The second concerns phosphorylation of the β1-adrenoceptor which blocks access to GαS. Note that adenylyl cyclase, substrates, and protein kinase-A are brought in close proximity by specialized scaffold proteins (AKAPs).

from an adjacent cell, the first target is the L-type Ca^{2+} channel CACNA1C. It is connected with PKA through AKAP6 or -7 and three phosphorylation sites have been identified, Ser1535, -1973, and -1981. Phosphorylation is associated with increased Ca^{2+} conductivity. This channel is functionally, and perhaps also physically, attached to a second Ca^{2+} channel, located in the membrane of the SR and named ryanodine receptor-2 (RYR2). The Ca^{2+}-influx across the plasmalemma opens RYR2 (it qualifies as a Ca^{2+}-induced Ca^{2+}-release channel) and this gives rise to an elevated level of intracellular free Ca^{2+}. The RYR2 channel has one PKA phosphorylation site, Ser2013, and its phosphorylation is associated with enhanced Ca^{2+}-sensitivity. Contraction commences as soon as intracellular free Ca^{2+} reaches 300 nm and maximum force is attained at 1500 nm (Figure 3-21(e)) (Bers, 2000). The entry of Ca^{2+} also sets in train the activity of a Na^+/Ca^{2+} exchanger (SLC8A1 or NCX), located in the plasmalemma, and a Ca^{2+}-pump located in the membrane of the SR (ATP2A2 or SERCA2) (accounting for 70% of the reuptake of Ca^{2+} in the human heart). The activity of this pump is controlled by phospholamban, a small transmembrane protein (only 52 residues). It forms a pentamer and its association with the Ca^{2+}-pump causes a distortion of the Ca^{2+}-binding sites with the consequence of a threefold reduction in transport at 300 nm free Ca^{2+} (Vostrikov et al., 2013; Akin et al., 2013). Phospholamban is connected to PKA via AKAP7 and phosphorylation at Ser16 causes a conformational change by which the complex loses its inhibitory control and Ca^{2+} is pumped more effectively. Enhanced uptake of Ca^{2+} reduces the rate of muscle relaxation (chronotropic effect) and prepares the heart better for a subsequent beat because the stores are fully recharged (inotropic effect) (MacLennan and Kranias, 2003). Another target is a voltage-gated K^+-channel (KCNQ1 complexed with KCNE1). It opens when the membrane depolarizes and phosphorylation at Ser27 increases its conductivity. The repolarization phase, therefore, will be more abrupt (Chen and Kass, 2011). PKA also interferes with Ca^{2+} binding to the troponin complex which is composed of troponin-I (binding to actin), troponin-C (Ca^{2+} binding), and troponin-T (binding to tropomysin). It sterically hinders myosin binding to actin filaments in the absence of Ca^{2+}. Troponin-I is phosphorylated on Ser23/24 which reduces the affinity for Ca^{2+} of troponin-C. This seems paradoxical because, at a given Ca^{2+} concentration, phosphorylation of troponin-I actually diminishes the number of cross-bridges between actin and myosin and thus weakens the contractile force. It is, however, likely that the increased levels of Ca^{2+} can easily overcome this problem. Importantly, the reduction in affinity makes sense in the perspective of contraction-duration because a reduced affinity translates in an increased off-rate and this means that the contraction phase is not unnecessarily lengthened by Ca^{2+} still bound to troponin-C. Remember that the heart can only effectively refill with blood after complete relaxation (diastole) and when rates go up to 180 beats/minute every fraction of a second counts!

If you wish to learn more about heart diseases (cardiomyopathies) read: Harvey and Leinwand (2011), Maron et al. (2006).

For a more concise overview of Ca^{2+} movements in the cardiac myocyte refer to Bers (2010).

For more information about HCN channels and their role in pacemaker activity in the heart and the brain refer to Postea and Biel (2011).

Effect of PKA on the SA node

The heartbeat rate will only effectively increase if the pacemaker cells augment their rhythm. Indeed, at the level of the SA node, β1-receptor-mediated increase in cAMP augments the conductivity of both the HCN4 channel, a cyclic nucleotide-gated Na^+/K^+ channel, thereby, increasing the inward Na^+ flow (If) and, through PKA-mediated phosphorylation, the conductivity of the Ca^{2+}-selective CACNA1G channel (T-type current). Together they give rise to more frequent action potentials. The HCN4 channel belongs to a family of cyclic nucleotide-gated ion channels (see also Figure 3-14(b)). They are composed of four identical subunits that constitute a cation channel. The cyclic nucleotide-binding domains in the cytoplasmic segment control the flow of ions. In the absence of cAMP (or cGMP), they obstruct the pore of the channel.

Retrocontrol mechanisms

The above-described events are transient because of several feedback mechanisms. The most important one is the intrinsic control of the action potential. The voltage-gated Na^+-channels (SCN5A) become refractory once the membrane potential has reversed to positive values (+20 mV). This refractory phase last for 240 ms and this prevents the summation of contraction waves without intermittent relaxation (which would stop circulation of the blood). While the increased outflow of K^+ ions (KCNQ1) slowly repolarizes the membrane, bringing it back to −90mV, the voltage-sensitive Ca^{2+} influx (CACNA1C) is also arrested and Ca^{2+}-pumps (ATP2A2, ATP2B2) and Ca^{2+}/Na^+ exchangers (SLC8A1) will reduce the intracellular free concentration to 100 nm (resting level). The production of cAMP is also arrested because of phosphorylation of the β1-adrenoceptor by PKA and, more rapidly and efficiently, by GRKs which causes the binding of arrestin and blocks interaction with adenylyl cyclase. Other pathways branch of from arrestin and these will be discussed below. Moreover, increased phosphodiesterase (PDE4) will remove the cAMP and levels return to normal. We return extensively to phosphodiesterases in Chapter 4 (regulation of muscle contraction by acetylcholine).

ARRESTING THE β-ADRENORECEPTOR SIGNAL: PATHWAY SWITCHING AND THE ROLE OF G-PROTEIN RECEPTOR KINASE AND ARRESTIN

Activation of Gs and subsequent functional separation of Gαs and Gβγ not only offers the opportunity to stimulate adenylyl cyclase, it also leads to recruitment of the G-protein coupled receptor kinase ADRBK1 (GRK2 or BARK1). The protein kinase, which is another G-protein effector, binds the Gαs with its RGS domain and it binds Gβ with its PH-domain. ADRBK1 belongs to a family of G-protein coupled receptor kinases, which, as a group (named "group G"), constitutes a subfamily within the large superfamily of RGS-box proteins (return to Chapter 2, Figure 2-31). GRKs also belong to the AGC protein kinase family which are grouped together because they share similar regulatory mechanisms (rendering the kinase catalytically competent) which were first identified for PKA, G (PKG), and C (PKC). Members of this family contain a typical C-tail which wraps around the N-lobe and the C-lobe (subject elaborated in Chapter X). The G-protein receptor kinases contain seven members, of which ADRBK1 and its closest homolog ADRBK2 distinguish themselves from the other members because of the aforementioned PH-domain (Figure 3-23). The other kinases do not interact with G-proteins but they do interfere with G-protein signaling (we return to this in the next section about biased agonists).

G-protein-mediated recruitment of ARKB1 to the membrane results in the phosphorylation of the C-terminal tail of the β1-receptor (the phosphorylation sites are still ill defined). This in turn creates a docking site for β-arrestin-1. The process by which arrestin binds the phosphorylated receptor is not fully elucidated but structure analysis of β-arrestin-1 bound to the C-terminal of the vasopressin receptor (AVP2R) has given important hints (Shukla et al., 2013). An initial complex is formed through interaction of arrestin with the phosphorylated receptor. This causes the displacement of its C-terminal sequence, an event that is followed by conformational changes of three loops (the finger, middle, and Lariat loops) that locate in the polar core region. Collectively these changes open the way for a second step, where the finger-loop of β-arrestin-1 docks into the pocket of the activated receptor. As a consequence, the access to G-proteins is blocked, the Gα-subunits gradually hydrolyze their GTP and turn into the inactive state (reforming the Gαβγ complex), and the production of cAMP is arrested. The receptor is said to be "desensitized" (process referred to as homologous desensitization) (Figure 3-24).

The liberated C-terminal segment of β-arrestin-1 (arrestin-2) interacts with the adaptor-protein-2 complex (subunit adaptin-2β or AP2B1) (Laporte et al., 2000) or with clathrin (N-terminal β-propeller domain) (Kim and Benovic, 2002; Kang et al., 2009). Both interactions

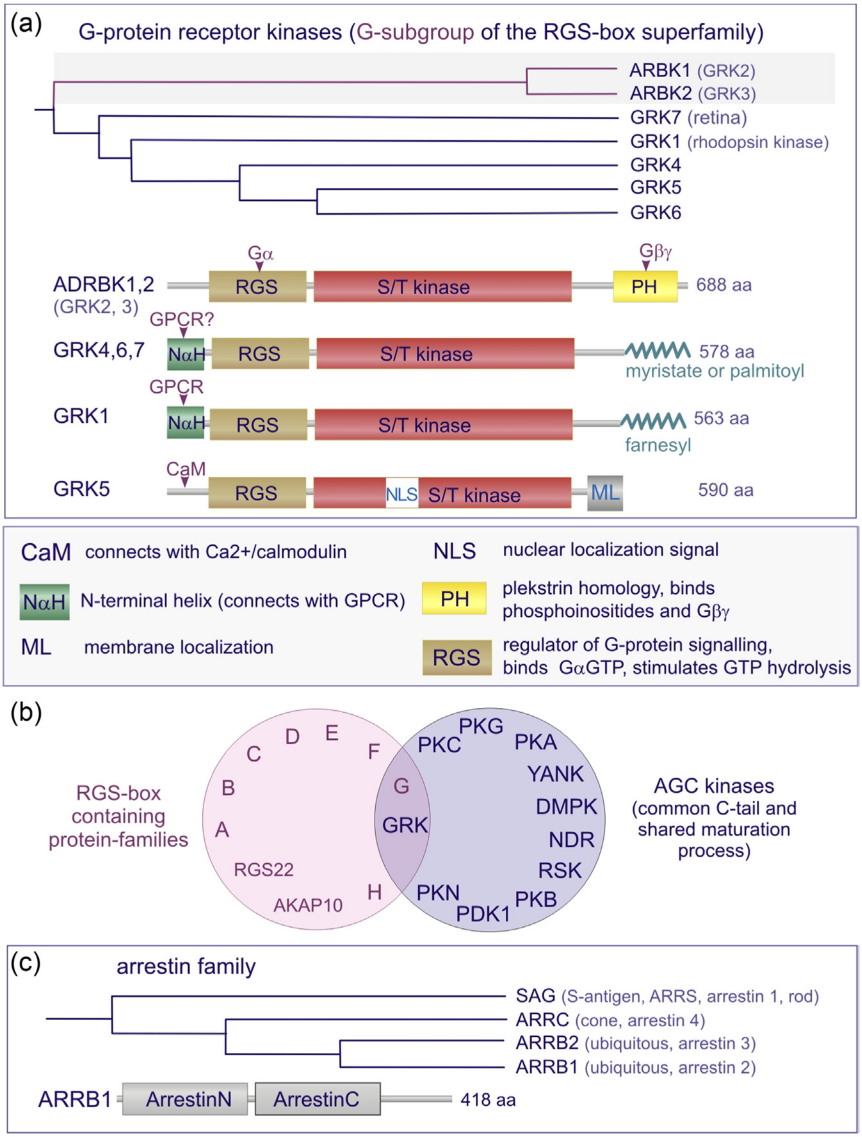
(a)
G-protein receptor kinases (G-subgroup of the RGS-box superfamily)
ARBK1 (GRK2)
ARBK2 (GRK3)
GRK7 (retina)
GRK1 (rhodopsin kinase)
GRK4
GRK5
GRK6
Gα
Gβγ
ADRBK1,2
(GRK2, 3)
RGS
S/T kinase
PH
688 aa
GPCR?
GRK4,6,7
NαH
RGS
S/T kinase
578 aa
myristate or palmitoyl
GPCR
GRK1
NαH
RGS
S/T kinase
563 aa
farnesyl
CaM
GRK5
RGS
NLS
S/T kinase
ML
590 aa
CaM connects with Ca2+/calmodulin
NLS nuclear localization signal
NαH N-terminal helix (connects with GPCR)
PH plekstrin homology, binds phosphoinositides and Gβγ
ML membrane localization
RGS regulator of G-protein signalling, binds GαGTP, stimulates GTP hydrolysis
(b)
RGS-box containing protein-families
A
B
C
D
E
F
G
GRK
H
RGS22
AKAP10
PKG
PKC
PKA
YANK
DMPK
NDR
RSK
PKB
PDK1
PKN
AGC kinases (common C-tail and shared maturation process)
(c)
arrestin family
SAG (S-antigen, ARRS, arrestin 1, rod)
ARRC (cone, arrestin 4)
ARRB2 (ubiquitous, arrestin 3)
ARRB1 (ubiquitous, arrestin 2)
ARRB1
ArrestinN
ArrestinC
418 aa

FIGURE 3-23 Domain architecture GRKs and arrestins. (a) The human genome contains seven members of the G-protein coupled receptor kinases (GRK). Note that the ARBK1 and -2 distinguish themselves by the possession of a PH-domain. This confers binding to Gβγ and explains why these two kinases are activated by heterotrimeric G-proteins. GRK1, -4, -6, and -7 possess a fatty acid or isoprenoid attachment to their C-terminus which associates them with the membrane (intrinsic membrane protein). The N-terminal segment of GRK5 binds the membrane in an as yet unclear fashion. For GRK1, exclusively expressed in cone/rod cells of the eye, the N-terminal α-helix (NαH) inserts into the activated rhodopsin receptor, very much in the same way the α5-helix of Gα-subunits docks onto GPCRs. Whether or not GRK4, -6, and -7 directly dock onto activated GPCRs remains to be proven, hence the question mark. (b) The G-protein-regulated protein kinases qualify as members of two families. They belong to the regulator of G-protein-signaling box (RGS box) containing proteins and they share characteristics with the AGC family of serine/threonine protein kinases. Named after PKA, PKG, and PKC, this kinase family is united through the possession of a characteristic C-tail and a shared "maturation" process (rendering the kinase competent). (c) The human arrestin family contains four members identified by an N-terminal and one C-terminal arrestin domain. SAG and ARR3 are expressed exclusively in the eye (retina), whereas the β-arrestins (ARRB1 and -2) are ubiquitously expressed.

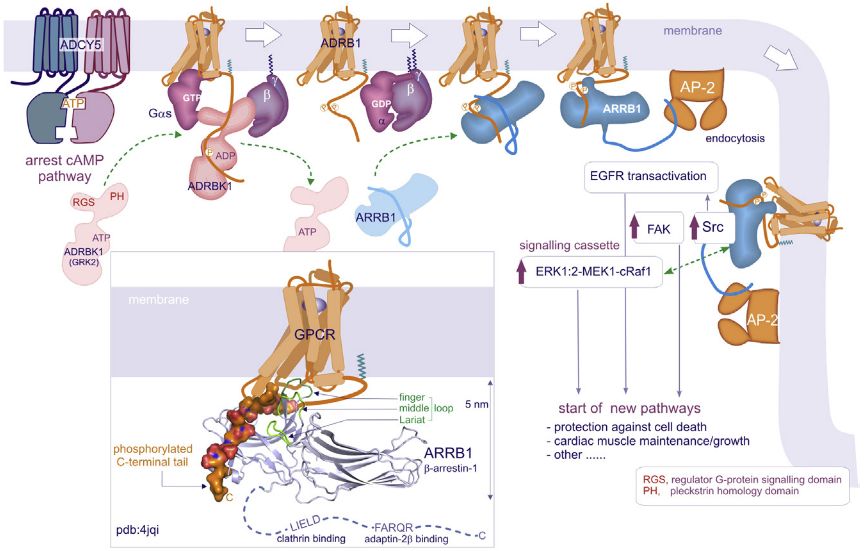

FIGURE 3-24 Pathway switching after binding of arrestin (ARRB1) to the β-adrenoceptor (ADRB1). Activated Gαs interacts with adenylyl cyclase (ADCY5 predominant in the heart) but also with G-protein-coupled receptor-kinase-2 (ADRBK1). The activated protein kinase phosphorylates residues in the C-terminal segment of the β1-adrenoceptor and this leads to binding and activation of β-arrestin-1. Interaction with the phosphorylated receptor initiates a series of conformational changes which start with the displacement of the C-terminal sequence, followed by a change in three loops at the polar core domain and this enables the docking of arresting into a pocket of the activated receptor (see image insert). Finally, depending on which sites of the receptor are phosphorylated, an as yet unknown set of conformational changes is instrumental in the binding of downstream signaling components (Src, cRaf1, and others). The displaced C-terminal segment interacts with the adaptor protein AP-2 (and/or with clathrin) thus allowing endocytosis of the receptor complex. It is generally considered that the new signaling components play a role in protecting the cell against death (apoptosis) and play a role in the maintenance and growth of the cardiac muscle.

conduct the receptors to an endocytotic pit (clathrin-coated) from which they are internalized into an early endosome (Menard et al., 1996). From here the receptor can return to the cell surface (recycling) or remain inside the cell to be degraded in a late endosome/lysosome. It is thought that the recycling mechanism is of particular importance in locations, such as sympathetic varicosities and synapses, where neurotransmitter concentrations have a tendency to soar. Transient removal of the receptor would avoid overstimulation. Trafficking of the receptor involves β-arrestin and it may serve as an adaptor, linking the receptor to several motor proteins (Kovacs et al., 2008). During the recycling process, the relative acidity of the early endosome helps to remove adrenaline, which must be removed from the vesicle by a transporter protein, and serine/threonine phosphatases

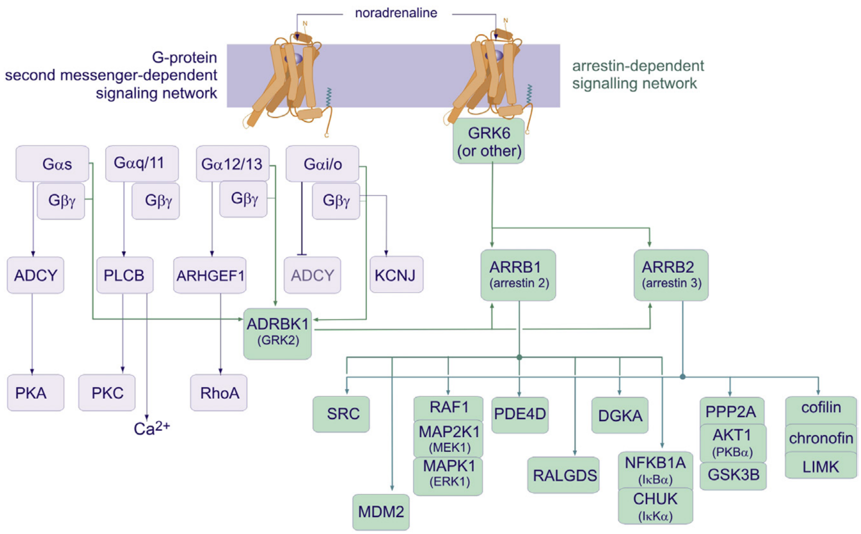

FIGURE 3-25 An explosion of signaling pathways. Noradrenaline binding leads to activation of different G-proteins (depending on receptor subtype). These induce or inhibit a series of signaling pathways and they cause the activation of ARKB1 (GRK2). Noradrenaline binding can also lead to direct interaction of the activated receptor with GRK6 (or perhaps other members of this family). Both GRK2 and GRK6 cause phosphorylation of the receptor (not necessarily on the same residues) and cause activation of β-arrestins. These arrest the G-protein-mediated events and serve as platforms for a whole new set of signaling pathways. *Figure adapted from Luttrell and Miller (2013).*

dephosphorylate the receptor, so that it arrives at the membrane in a resensitized state. PPP2CA is a good candidate for the dephosphorylation reaction but it is not clear yet which of the 15 regulatory subunits of this catalytic subunit mediates β-adrenoreceptor specificity (return to Chapter 2, Figure 2-47) (Vasudevan et al., 2011).

Whether internalized or not, arrestins serve as a scaffold to which a number of other signaling components bind (see Figure 3-25) (Xiao et al., 2007, 2010; Luttrell and Miller, 2013). The term scaffold implies that arrestin acts as a platform to which a specific set of proteins bind, forming a cassette, each of which interacts with each other in order to relay the receptor-signal into the cell. Scaffolding proteins perform at least three functions in cells: they (1) increase the efficiency of signaling between successive components of an enzymatic cascade, (2) ensure signaling fidelity by dampening cross talk between parallel cascades, and (3) localize signaling to specific subcellular locations (Burack and Shaw, 2000). It is not clear yet how the signaling components interact with arrestins and whether or not different components can attach to the same arrestin. The remarkable diversity of signaling pathways that emanate from arrestins suggests that they may assume a variety of conformations with distinct functional capacities. How ligand/receptor interaction mediates distinct conformational changes is not known (Shukla et al., 2011; Lefkowitz, 2013). Within the context of the cardiomyocytes and β-arrestin-1, the pathway switch has a long-term impact on the organization of the contractile units, the number of cells, and their survival. The first protein kinase to be found associated with β-arrestin-1 was c-Src, which operates in growth factor-signaling pathways (Luttrell et al., 1999). It was shown that c-Src, through stimulation of membrane-bound metalloproteases, enables the liberation of epidermal growth factor (EGF), thus initiating a genuine growth factor signal (Prenzel et al., 1999). β-arrestin-1 also binds a protein kinase cassette comprising cRaf1-MEK1-ERK1/2, a signaling pathway that acts downstream of numerous growth factor receptors and is intimately related to cell survival and cell proliferation. We return to a number of these arrestin-induced pathways in the following chapters.

In Figure 3-3, we distinguish the different adrenoceptors by the G-protein they engage. We could add to this list, albeit it unconventional, GRKs and β-arrestins as they too play the role of signal transducers. In the case of GRKs, with the exception of ADRBK1 and -2, they probably directly compete with binding of G-proteins to the receptor. Relative concentrations of GRKs and G-proteins must, therefore, be a critical component in signal outcome. The above described processes operate in many GPCR and arrestin-initiated signaling events are common to all of them.

DISCOVERY OF ARRESTIN

The first arrestin was discovered in experiments with isolated disc membranes from bovine retinal rod-photoreceptor cells to which purified cGMP-phosphodiesterase, ATP, and an unknown protein of 48 kDa were added. The choice of the protein was not random, it had sparked interest because it was recovered from the cytosol under dark conditions but was found associated with the disc membrane under light conditions. Addition of the 48-kDa protein, under conditions suitable for protein phosphorylation, greatly diminished the activation of cGMP-phosphodiesterase (enzyme that converts the second messenger, cyclic GMP into GMP) (Kuhn, 1978). The authors wrote in their discussion: *Our results show that 48-kDa protein, in combination with phosphorylation of the receptor, quenches ("arrests") the ability of the receptor to activate phosphodiesterase, most probably because 48-kDa protein binds to the phosphorylated receptor and thereby competes with transducin (G-protein). The name "arrestin" therefore seems appropriate…* (Wilden et al., 1986). Only later it became clear that rod arrestin corresponded to a 50-kDa protein, that was long known to be the target

DISCOVERY OF ARRESTIN *(cont'd)*

of antibodies in experimental uveitis (inflammation of the retina) and was named "soluble antigen" or S-antigen (Pfister et al., 1985). This explains why the official gene name for rod-arrestin is SAG (abbreviation of S-antigen). About four years later, it was shown that an analogous protein arrests adrenoceptor-mediated activation of Gs (as measured by hydrolysis of ^{32}P-GTP). The newly discovered member of the arrestin family (tentatively named β-arrestin) arose from a screening of a bovine brain cDNA library with the DNA sequence of bovine p48 which was just released (Shinohara et al., 1987; Benovic et al., 1987; Lohse et al., 1990).

Web resource about AGC kinase group http://www.cellsignal.com/reference/kinase/agc.html.

Homologous versus heterologous receptor desensitization

A number of serines and threonines of the GPCR are also substrate of PKA and their phosphorylation interferes with G-protein activation, hence leading to receptor desensitization. Figure 3-5 shows an example of PKA phosphorylation sites on the β2-adrenoceptor. Unlike GRKs, which require interaction with the activated receptor and, therefore, only affect those receptors that are signaling, referred to as homologous desensitization, PKA can reach any receptor and cause their phosphorylation. The process whereby one ligand attenuates the response of other nonrelated ligands is called heterologous desensitization. It has been suggested that on the long run, PKA activity may also increase GRK2 and β-arrestin-1/2 expression, adding an extra level of (long-term) retrocontrol. Moreover, another protein kinase, PKC, activated by DAG and Ca^{2+}, and thus downstream of G-proteins that interact with PLC-β, may facilitate the activation of GRK2. Both mechanisms lead to an enhanced homologous desensitization (review Chuang et al., 1996).

To illustrate how GRK2 and PKA feedback loops affect signal outcome in the cardiomyocytes, we show the results of a computer-modeling approach in which the parameters are, as much as possible, based on experimental results. The model features two feedback loops, one emanating from GRK2, a very short loop, the other from PKA, a loop with many intermediate steps. Parameters are activation of Gαs, production of cAMP, and phosphorylation of phospholamban (voir Figure 3-22). Noradrenaline is added for 40 s, at a concentration that causes maximum stimulation of Gαs and a near-maximum production of cAMP. The respective activities are presented as fractional activation, where 1.0 represents the maximum activity any individual component could attain (Figure 3-26). The simulation reveals that none of the parameters reaches their maximum level, all activities oscillate around 50%, in other words, negative feedback controls output. Oscillation is caused by time delay, where the system is turned fully on, then with a delay turns on the negative feedback, which in the process of inhibition, turns itself off and the cycle repeats as long as the stimulus (adrenaline) is present.

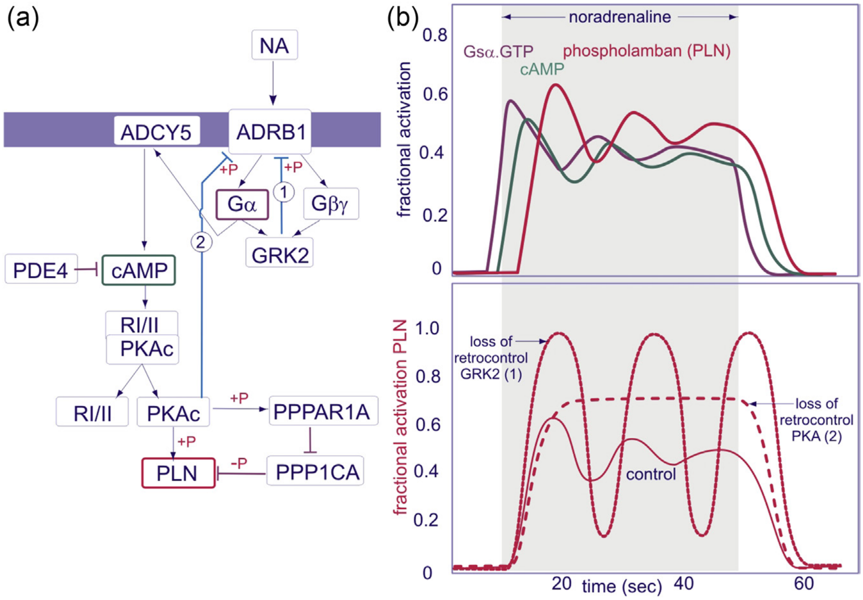

FIGURE 3-26 Computer simulation of noradrenaline-mediated activation of signaling events in cardiomyocytes. (a) Graph illustrating the activation of Gαs (exchange of GDP for GTP), production of cAMP, and phosphorylation of phospholamban (regulatory subunit of ATPA2 (SERCA2)) after the addition of noradrenaline at a concentration which causes maximum Gαs activation and a near-maximum cAMP production (maximum value set at 1.0). Note that none of the parameters reaches their maximum, the response oscillates around 50% which means that the system is controlled by inhibitory feedback. Removal of noradrenaline leads to a quick return to basal levels of all parameters. (b) To distinguish between the action of GRK and PKA, each of the feedback loops was disrupted. Loss of PKA-feedback results in a dampened steady-state level of phospholamban phosphorylation. This can be explained by a rapid, almost instantaneous, negative feedback by GRK. On the contrary, disrupting the GRK-feedback causes strong oscillations with near-maximum responses at each cycle. This profile is characteristic of a delayed feedback system. The oscillations in phospholamban phosphorylation are the consequence of oscillating PKA activities against a background of phosphatase activity which increases with low PKA activity and decreases with high PKA activity (not shown). The weak oscillations in the control situation thus are the consequence of a steady-state control by GRK2 and an oscillatory control by PKA. *The graphs are adapted from Kraetler et al. (2010).*

In dissecting the relative contribution of GRK2 and PKA, simulations were performed in which each of the feedback loops were disrupted (corresponding reaction-weights were put to zero). The simulation shows two quite different outcomes for the phosphorylation of phospholamban. In the absence of PKA, the response is dampened without oscillations. This can be explained by the rapidity of GRK2 feedback loop, the almost instantaneous shut off creates a steady level of diminished signal activity. Removing GRK2 gives rise to very strong oscillations which can be explained by the longer time delay between receptor activation and PKA-mediated inhibition. The oscillations in phospholamban phosphorylation are the consequence of oscillations in PKA activity, against a background of phosphatase activity. The two different feedback characteristics explain why in the control situation you have an oscillating (PKA input) and dampened response (GRK2 input) (Figure 3-26) (Kraetler et al., 2010).

Detrimental effects of adrenoreceptors signaling; a protective role of biased agonists (beta-blockers)

Excessive sympathetic control (elevated levels of noradrenaline) is one of the characteristics of a late-stage cardiac myopathy (condition of heart failure). Under this condition, it impairs diastolic function, causes tachycardia (fast beat) and tachyarrhythmia (contractions of atria and ventricles are not coordinated) and this leads to myocardial ischemia, followed by stunning (persistent contractile dysfunctioning after transient ischemia) and ultimately cell death (infarction). The detrimental effects of sympathetic control are dependent on adenylyl cyclase type 5 (ADCY5) and are associated with elevated levels of GRK2 expression. Somehow elevated levels of GRK2 annihilate the growth and survival signals that emanate from receptors of growth regulatory cytokines. On the contrary, the signaling pathway of the TNFα-receptor is boosted and this may explain the occurrence of cell death (Harvey and Leinwand, 2011; Pyle and Solaro, 2004; Vasudevan et al., 2013; Fu et al., 2013; Noma et al., 2007; Cannavo et al., 2013; Zaugg et al., 2000).

The above-described action of noradrenaline and adrenaline must be seen against the background of a multitude of soluble messengers that perfuse the heart and that play essential roles in its development but also in maintenance and survival of cardiac myocytes. Among these messengers, we cite growth factors (insulin, EGF, neuregulin-1), inflammatory mediators (TNFα), hormones (angiotensin, endothelin, vasopressin, adrenaline), and we include components of the extracellular matrix that surround the plasmalemma (Figure 3-27). The protein kinase AKT2 plays an essential role as a downstream effector of a number of these mediators because it is a key player in the prevention of cell death (we return to this mechanism in detail in Chapters 11 “Signal Transduction to and from Adhesion Molecules” and 16 “Signaling through the Insulin Receptor: Phosphoinositide 3-Kinases and AKT”) (Bertrand et al., 2008).

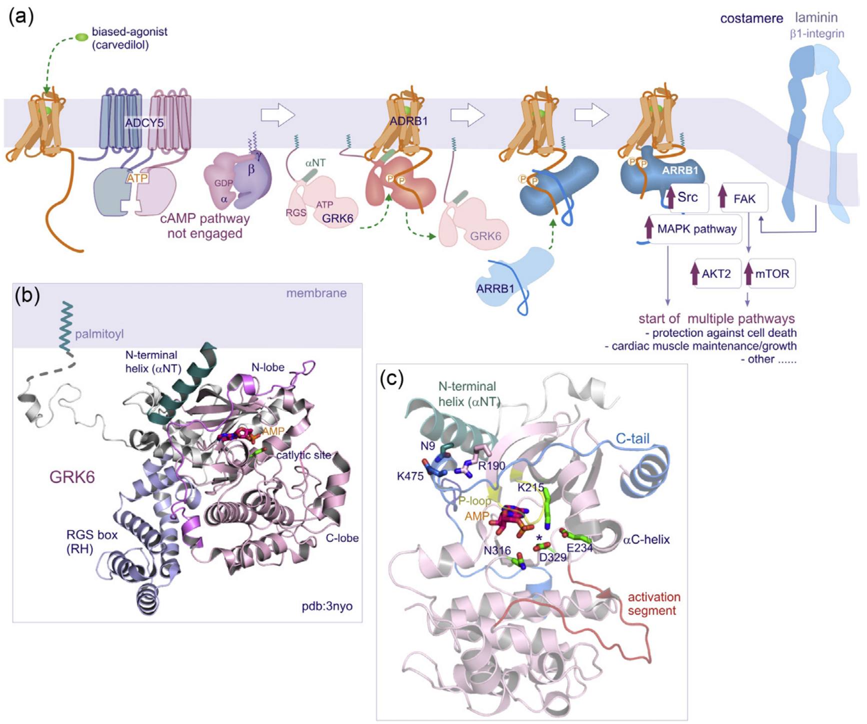

FIGURE 3-27 The alleged protective mechanism of the biased agonist carvedilol on cardiomyocytes. (a) Carvedilol does not engage the cAMP-pathway and that's why it originally qualified as a beta-blocker. In the case of critical heart failure, which is accompanied by a high sympathetic tone, the beta-blocker action protects the heart from cell death caused by incessant high concentrations of noradrenaline. Carvedilol qualifies as a biased agonist because it does engage β-arrestin-1 (ARRB1) with the help of GRK6 (and not GRK2). This leads to the activation of the MAPK pathway. Other pathways may also be involved, like the one emitting from focal adhesion kinase and involving AKT2 and mTOR. These are known to be involved in the extracellular matrix signaling and play an important role in survival of epithelial cell. We speculate that myocyte adhesion, at special regions, named costameres, also plays an important role in all this. (b) Molecular structure of GRK6, showing the C-terminal palmitate, the RGS-box (sometimes referred to as RGS-homologous or RH domain), the protein kinase domain with its C- and N-lobe, and the N-terminal helix. The latter is thought to fit into the pocket of activated GPCRs, in a manner similar to the insertion of the α-5 helix of Gαs. (c) Focus on the molecular structure of the kinase domain of GRK6 to illustrate that the kinase is in a near-competent configuration. The RGS-box and part of the C-terminal region have been removed. GRK6 belongs to the large family or AGC protein kinases which are characterized by a C-terminal segment (C-tail, in blue) that wraps around the N- and C-lobe and plays a critical role in regulation of kinase activity. The N-terminal helix, the C-tail, and the N-lobe make contact, as illustrated by the hydrogen bonds that form between the conserved N9, K475, and R190 residues respectively. Like the ACG kinases, GRK does not require extensive changes for expressing its catalytic activity. The three highly conserved residues that coordinate the adenosine nucleotide are already in the right position (K215, E234, and N316) and the activation loop is fully structured and outward oriented. D329* is the catalytic residue. It is not yet clear whether additional "activating" conformational changes occur upon docking of GRK6 into the pocket of the activated adrenoceptor. Pdb: 3nyo.

Beta-blockers are beneficial in the condition of critical heart failure, in part, because they reduce the excessive sympathetic control but for carvedilol, a biased agonist (Figure 3-8 and 3-10), other mechanisms are in place. Carvedilol is one of the beta-blockers for which it has been shown that it effectively reduces mortality over a period of 12 months (Chatterjee et al., 2013; Zhu et al., 2001). As we have mentioned earlier in this chapter, besides an arrest of cAMP production, it also causes activation of the mitogen-activated kinases (MAPK or ERK1/2), a protein kinase normally engaged by growth factors when cells are attached to the extracellular matrix. Carvedilol does not signal via Gαs and, therefore, does not recruit GRK2 to the membrane. Instead, it causes activation of GRK6 (Figure 3-27). Although still speculative, GRK6, which is attached to the membrane by a farnesyl chain, probably directly binds to the active β1-adrenoceptor (or α1/β2-receptors) and phosphorylates residues in the C-terminal. This has been studied in detail for the β2-receptor in which it was shown that GRK6 phosphorylates residues distinct from GRK2 or PKA (see sequence insert of Figure 3-5(a)) (Nobles et al., 2011). Like for GRK2, this leads to recruitment of β-arrestins (ARRB1 or ARRB2) and subsequent attachment of effector enzymes. From here a cascade of events sets off, involving focal adhesion kinase among others and that ultimately avoid cell death (or make it less likely to occur) (Figure 3-25) (Luttrell and Miller, 2013). It is has been shown that myocyte adhesion to extracellular matrix also plays an important role in myocyte growth and survival. Integrins signal through cytoplasmic protein kinases such as integrin-linked kinase and focal adhesion kinase (PTK2, PTK2B) (Pentassuglia and Sawyer, 2012; Amin et al., 2011). Adhesive contacts occur at specialized regions, named costameres, where the extracellular matrix is connected with the sarcomere (at the level of the Z-disks). Adhesive contacts play an important role in mechanotransduction, they relay stretching of the muscle with increased contractile force by enhancing cAMP production (Todaka et al., 1998). We speculate that costamere signaling teams up with the adrenoceptor biased signal in order to help making the myocyte more resilient to unfavorable conditions (Franchini, 2012; Pentassuglia and Sawyer, 2013). Cooperation between growth factors and integrins are vital for the survival of epithelial cells (Frisch et al., 1996).

Arrestin-mediated events are similar but not identical to growth factor-mediated events

Arrestins are platforms for a number of signaling components that are also recruited by growth factor receptors and, therefore, mimic growth factor effects. But this is not the whole story. The cRaf1-MEK1-ERK1/2 cassette operational downstream of, for instance, the EGF receptor, leads to detachment and subsequent nuclear translocation of ERK1/2 where it phosphorylates transcription factors. This is an essential process to bring about cell division. When studying ERK activation in response to angiotensin-II, it was shown that the signal remains in the cytosol and has quite different cellular consequences (Ahn et al., 2004). Moreover, the signal is generally weak but long lasting. A possible implication of an excess of arrestin-signaling complexes could be the cytosolic sequestration of signaling components that normally would operate in the nucleus. This is perhaps one reason why an excess expression of ADRBK1 (GRK2), as occurs in late stages of heart failure, is lethal for cardiomyocytes. It may deprive the cardiomyocytes of appropriate regulation of gene expression that would normally allow them to withstand the rather aggressive conditions (Fu et al., 2013).

Heart failure, also known as congestive heart failure or cardiac myopathy, occurs when the heart muscle doesn't pump blood as well as it should. Conditions such as a narrowed coronary artery or high blood pressure leave the left ventricle of the heart too weak (dilated cardiomyopathy) or to stiff (hypertrophic myopathy) to fill up and pump efficiently. Symptoms are fatigue (quickly out of breath), water in lungs (because the right ventricle still works fine), and swollen ankles (because the blood is not properly pumped upward). Elevated sympathetic control, high levels of noradrenaline, is associated with advanced stages of heart failure and this is thought to play a role in cardiomyocyte death. Areas of dying cells cause conduction errors, they slow down progress of the action potential and when they enter healthy tissue they find myocytes that are no longer refractory. These inappropriate "reentry currents" cause rapid ventricular contractions which are no longer in phase with the rhythmicity of the SA–AV nodes (phenomenon known as flutter or fibrillation of the heart, a condition where the heart works but the blood does not really circulate). The employment of electroshocks causes a synchronized refractoriness of all excitable cells and this type of acute treatment rescues the heart if the SA node is able to impose a new, coordinated, rhythm. The elevated sympathetic tone generally reduces with mild aerobic exercise. Its impact on the heart can be reduced by the use of beta-blockers.

POTENTIAL THERAPEUTICAL USE OF BIASED AGONISTS IN PAIN RELIEF

The dual receptor response, G-protein-mediated versus arrestin-mediated, can be exploited for therapeutical purposes in the relief of pain. Morphine is still a most powerful analgesic and, of the four different opioid receptors, the µ-opioid (*m*-receptor or mu-receptor, named after morphine) receptor is most effective in this respect. However, adverse effects, such as

POTENTIAL THERAPEUTICAL USE OF BIASED AGONISTS IN PAIN RELIEF *(cont'd)*

constipation, nausea, and respiratory depression, as well as tolerance to the medicament, are not uncommon. These adverse effects are likely the consequence of β-arrestin-2-mediated signaling and not of the Gi-mediated pathway (inhibition of adenylyl cyclase by Gαi, increase in outward K^+ current by Gβγ). Searching for a biased agonist that engages Gαi but not GRKs could provide an improved therapeutic benefit (Kelly, 2013).

α1-ADRENOCEPTORS AND VISCERAL VASOCONSTRICTION

Initiation of contraction

Increased cardiac output (increased stroke volume), which occurs under sympathetic control, requires rapid filling of the atria and this is dependent on the diastolic venous pressure. It, therefore, seems logic that noradrenaline (and adrenaline) brings about vasoconstriction but not everywhere. Both the heart itself and muscles, which are great oxygen consumers when working hard, need all the blood they can get, on the contrary, the digestive system and skin may do with less. Vasoconstriction is brought about by circular smooth muscle cells in response to the local liberation, from nerve varicosities, of noradrenaline. The contraction is slow and long lasting, in the order of half a minute, and if the presence of noradrenaline persist, the smooth muscle remains contracted (Figure 3-28(a)). Smooth muscle cells of arteries and arterioles carry predominantly α1-adrenoceptors (ADRA1A, -B, -D) and these are coupled to Gαq or Gα11 (Figure 3-28(b)). In essence, the Gαq pathway causes contraction, the Gα11 pathway sensitizes the contraction machinery. The process of excitation–contraction coupling involves the production of inositol-1,4,5-trisphosphate (IP_3) and the membrane-resident DAG both derived from cleavage of phosphatidyl-4,5-inositol bisphosphate (PIP_2). IP_3 liberates Ca^{2+} from intracellular stores through binding to a tetrameric Ca^{2+} channel (Figure 3-29). The release of Ca^{2+} is Ca^{2+}-facilitated, meaning that binding of IP_3 alone is not sufficient (Taylor et al., 2004). The liberated Ca^{2+} binds calmodulin, which binds myosin light-chain kinase (MYLK) and the ensuing change in conformation, removal of an inhibitory constraint, leads to kinase activation. Myosin light-chain kinase phosphorylates the regulatory subunit of myosin (MYL12B) and this sets in train the actin–myosin cross-bridge cycle (Figure 3-30). The Ca^{2+} store is replenished by

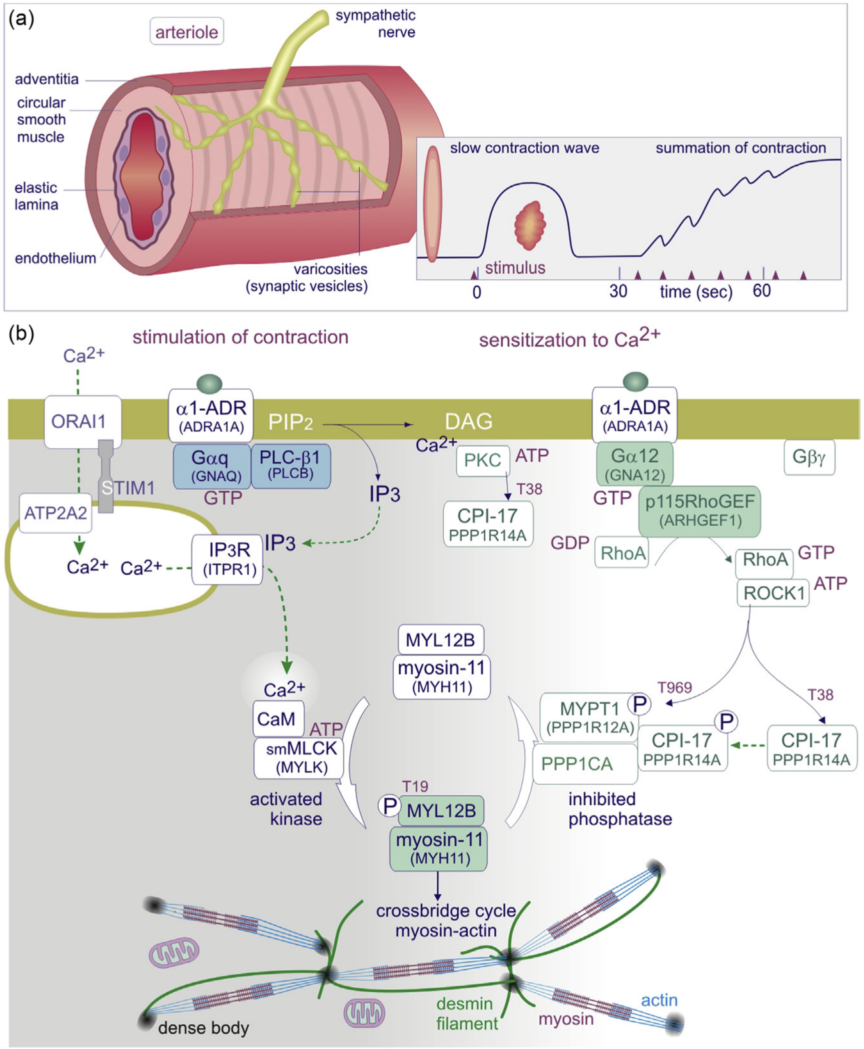

FIGURE 3-28 Arterial constriction induced by noradrenaline. (a) Sympathetic innervation of a small arteriole. Arterioles are composed of an endothelial lining, an elastic lamina, a circular smooth muscle cell layer, and an adventitia (connective tissue). Sympathetic nerve endings surround the smooth muscle cells and release noradrenaline at discrete sites, varicosities, from where it diffuses into the tissue. Smooth muscle contraction is slow and, with a single stimulus, lasts for some 30 s. Repeated exposure to adrenaline causes a permanent contractile state. (b) Smooth muscle cells in the viscera express the α1-adrenoceptor (ADRA1A) which is coupled to Gαq but can also be coupled with Gα12. The Gαq pathway induces contraction, whereas the Gα12 pathway sensitizes the contractile machinery for Ca^{2+}. Cαq activates PLCβ1 (PLCB) causing the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). This binds the IP_3 receptor located on the endoplasmic reticulum and causes the liberation of Ca^{2+}. As a consequence, myosin light-chain kinase is activated; it phosphorylates the regulatory subunit, a modification that unlocks the actin–myosin cross-bridge cycle. In parallel, the membrane-bound DAG, together with Ca^{2+}, activates protein kinase C and this causes phosphorylation of the phosphatase inhibitor CPI-17. The Gα12 pathway recruits the RGS-containing guanine-exchange protein p115RhoGEF (ARHGEF1) and activates RhoA. This in turn activates the serine/threonine protein kinase ROCK1 which phosphorylates MYPT1 (PP1R12A) and CPI-17 (PPP1R14A). Both substrates disable the action of PPP1CA leaving the kinase to dominate the phosphorylation/dephosphorylation cycle. The system is said to be sensitized to Ca^{2+}. Of note, Ca^{2+} stores are replenished by a Ca^{2+}-ATPase (ATP2A2) which takes source from Ca^{2+} that enters through a store-operated Ca^{2+} channel (ORAI1). STIM1 serves to measure Ca^{2+} levels.

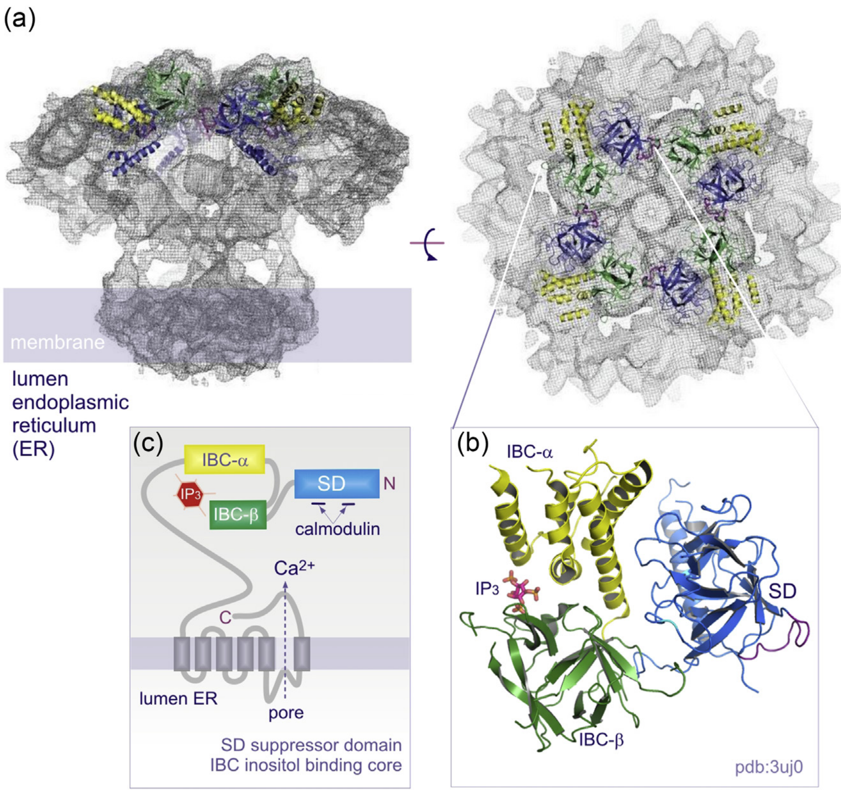

FIGURE 3-29 Detail of the IP_3-receptor. (a) Three-dimensional structure of the type 1 inositol-1,4,5-trisphosphate (IP_3) receptor at electronmicroscopic resolution (tomographical reconstitution). The receptor is composed of four identical subunits with a large cytoplasmic and a small transmembrane segment. The four subunits form a single Ca^{2+} pore. The colored elements represent the structure of the N-terminal region, comprising the suppressor domain (SD) and the IP_3-binding cores, IBC-α and IBC-β. *(Image from Seo et al. (2010).* (b) Detail of the N-terminal segment with SD, IBC-α, and IBS-β. IP_3 binds both IBC domains and brings about a change in the position of the SD. This domain binds calmodulin and it is suggested that the SD/calmodulin complex controls gating of the Ca^{2+} pore. Of note, ion flow requires the presence of both IP_3 and Ca^{2+}. Pdb:3uj0. (c) Membrane topology of the IP_3 receptor.

a Ca^{2+}-pump (ATP2A2) which takes its source from the Ca^{2+} that enters through a storage-operated Ca^{2+} channel (ORAI1). This channel senses Ca^{2+} levels in the intracellular store through a connection with STIM1 (Figure 3-28).

Sensitization of smooth muscle to Ca^{2+}

Under different experimental conditions, it was shown that with identical intracellular Ca^{2+} concentrations, the force of muscle contraction

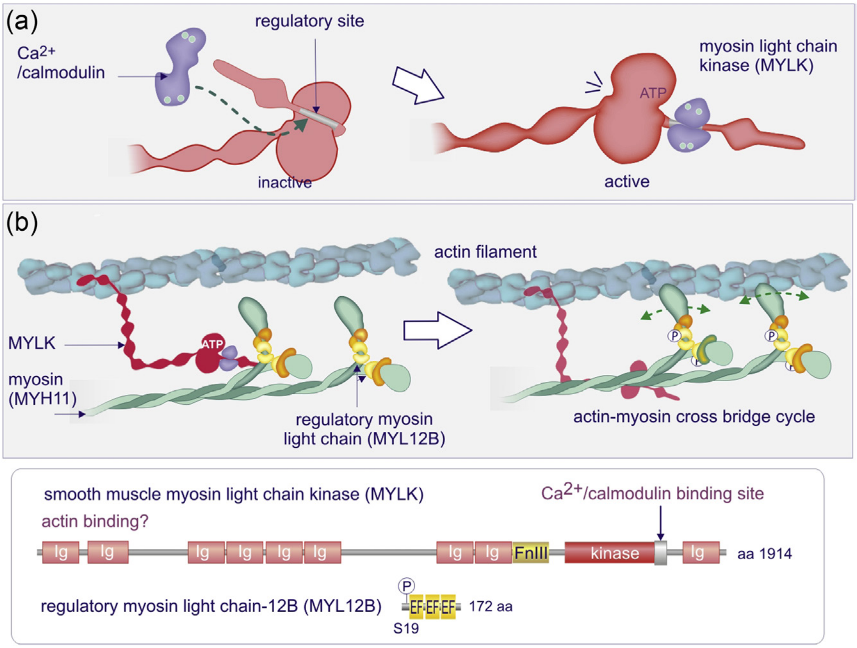

FIGURE 3-30 Detail of the myosin light-chain kinase activation mechanism. (a) Upon entering the cytoplasm, Ca^{2+} binds to calmodulin which brings about a conformational change. The Ca^{2+}/calmodulin complex binds and displaces an autoinhibitory region of myosin light-chain kinase (MYLK) and this constitutes its activation. (b) Myosin light-chain kinase phosphorylates the regulatory subunit of smooth muscle myosin (MYL12B) and this sets in train the actin–myosin cross-bridge cycle. Myosin light-chain kinase is composed of a series of immunoglobulin-like (Ig) domains, one of which must assure its binding to actin. The regulatory myosin light-chain is composed of three EF-hand domains and is phosphorylated on Ser19.

could vary, as if the contraction machinery was more or less sensitive to Ca^{2+}. Indeed, a parallel pathway is operational in smooth muscle which acts on the phosphatase that normally removes the phosphate from the regulatory subunit of myosin. If its activity is high, the cells will be less sensitive, they require more Ca^{2+} to maintain contraction, and the opposite is true for an inhibited phosphatase. The smooth muscle phosphatase in question is composed of a catalytic subunit, PPP1CA, and a regulatory subunit MYPT (PPP1R12A). The regulatory subunit serves to make the PPP1CA highly substrate-specific; it exclusively dephosphorylates the myosin light-chain protein. Activity of the phosphatase can be controlled by the binding of an inhibitor CPI-17 (PPP1R14A) which sterically hinders access of substrate. Its potency to inhibit PPP1CA increases 1000-fold by phosphorylation on Thr38 (Ohki et al., 2001; Eto et al., 2007). Modification of the regulatory subunit, through phosphorylation on Thr969, also inhibits phosphatase activity.

Two protein kinases play a role in the phosphatase inhibition process. The first is protein kinase C, which is activated by DAG and Ca^{2+} and it phosphorylates the inhibitor CPI-17(PPP1R14A). The other is ROCK1. The activation pathway involves the α1-adrenoceptor but this time coupled to Gα12. In its GTP-bound state it interacts with p115RhoGEF (ARHGEF1), an RGS-containing protein (like the GRKs) but this time coupled with a guanine-nucleotide exchange domain (GEF) which interacts with the monomeric G-protein RhoA (for domain composition of the RGS family of proteins return to Chapter 2, Figure 2-31). In its GTP-bound state, RhoA interacts and activates Rho kinase-1 (ROCK1) which in turn phosphorylates CPI-177 (PPP1R14A) and MYPT (PPR1R12A). As a consequence of activation of both PKC and ROCK, the smooth muscle cell lacks PPP1CA activity and this explains why intermittent waves of Ca^{2+} suffice to keep the cell in a contracted state. Naturally, Gαq- and Gα12-mediated signaling is dampened by the intervention of GRKs, which after phosphorylation of the α1-adrenoceptors and recruitment of β-arrestins will block the activation of PLCβ (subject not further elaborated).

Web resource

KEGG map 04270 http://www.genome.jp/kegg/kegg2.html.

ADRENALINE (AGAIN)

> It was ever thus. The cab-choked street, the PR men clutching their ulcers, the jewellery displayed like medals on the chest of a Soviet general, the snoozing men from Wall Street, the Sardi's supper entrance. As always in any enterprise, Americans travel hopefully, fuelled by a thirst for adrenalin not experienced by most Europeans
> ***John Osborne Almost a Gentleman (Faber & Faber, London, 1991)***

References

Ahlquist, R.P., 1948. A study of the adrenotropic receptors. Am. J. Physiol. 153, 586–600.

Ahn, S., Shenoy, S.K., Wei, H., Lefkowitz, R.J., 2004. Differential kinetic and spatial patterns of β-arrestin-2 and G protein-mediated ERK activation by the angiotensin II receptor. J. Biol. Chem. 279, 35518–35525.

Ahlquist, R.P., 1973. Adrenergic receptors: a personal and practical view. Perspect. Biol. Med. 17, 119–122.

Akin, B.L., Hurley, T.D., Chen, Z., Jones, L.R., 2013. The structural basis for phospholamban inhibition of the calcium pump in sarcoplasmic reticulum. J. Biol. Chem. 288, 30181–30191.

Amin, P., Singh, M., Singh, K., 2011. β-adrenergic receptor-stimulated cardiac myocyte apoptosis: role of β1-integrins. J. Signal Trans. article ID 179057.

Artymiuk, P.J., Poirrette, A.R., Rice, D.W., Willett, P., et al., 1997. A polymerase I palm in adenylyl cyclase? Nature 388, 33–34.

Ballesteros, J.A., Weinstein, H., 1995. Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. Methods Neurosci. 25, 366–428.

Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Caron, M.G., Lefkowitz, R.J., 1987. Functional desensitization of the isolated b-adrenergic receptor by the β-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). PNAS U.S.A. 84, 8879–8882.

Berridge, K.C., 2006. The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl.) 191, 391–431.

Bers, D.M., 2010. Calcium fluxes involved in control of cardiac myocyte contraction. Circ. Res. 87, 275–281.

Berstein, G., Blank, J.L., Jhon, D.Y., Exton, J.H., Rhee, S.G., Ross, E.M., 1992. Phospholipase C-β1 is a GTPase activating protein for Gq/11, its physiologic regulator. Cell 70, 411–418.

Bertrand, L., Horman, S., Beauloye, C., Vanoverschelde, J.L., 2008. Insulin signalling in the heart. Cardiovasc. Res. 79, 238–248.

Betke, K.M., Wells, C.A., Hamm, H., 2012. GPCR mediated regulation of synaptic transmission. Prog. Neurobiol. 96, 304–321.

Black, J.W., Leff, P., 1983. Operational models of pharmacological agonism. Proc. R. Soc. Lond Ser. B 220, 141–162.

Black, J.W., Crowther, A.F., Shanks, R.G., Smith, L.H., Dornhorst, A.C., 1964. A new adrenergic betareceptor antagonist. Lancet 1, 1080–1081.

Black, J.W., 2010. Reflections on drug research. Br. J. Pharmacol. 161, 1204–1216.

Bolwell, G.P., Cyclic, A.M.P., 1995. The reluctant messenger in plants. Trends Biochem. Sci. 20, 492–495.

Bond, M., Somlyo, A.V., 1982. Dense bodies and actin polarity in vertebrate smooth muscle. J. Cell Biol. 94, 404–413.

Bond, R.A., Leff, P., Johnson, T.D., Milano, C.A., Rockman, H.A., McMinn, T.R., Apparsundaram, S., Hyek, M.F., Kenakin, T.P., Allen, L.F., Lefkowitz, R.J., 1995. Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. Nature 374, 272–276.

Burack, W.R., Shaw, A.S., 2000. Signal transduction: hanging on a scaffold. Curr. Opin. Cell Biol. 12, 211–216.

Busti, S., Coccetti, P., Alberghina, L., Vanoni, M., 2010. Glucose signalling-mediated coordination of cell growth and cell cycle in *Saccharomyces cerevisiae*. Sensors 10, 6195–6240.

Cannavo, A., Liccardo, D., Koch, W.J., 2013. Targeting cardiac β-adrenergic signalling via GRK2 inhibition for heart failure therapy. Front. Physiol. 4 article 264.

Cannon, W.B., 1929. Bodily Changes in Pain, Hunger, Fear and Rage. An Account of Researches into the Function of Emotional Excitement. Reprinted, Harper Torch Books, 1963, New York.

Chatterjee, S., Biondi-Zoccai, G., D'Ascenzo, F., Castagno, D., van Tassell, B., Mukjerjee, D., Lichstein, E., 2013. Benefit of β blockers in patients with heart failure and reduced ejection fraction: network meta analysis. BMJ 346, 1–10.

Chen, L., Kass, R.S., 2011. A-kinase anchoring protein 9 and IKs channel regulation. J. Cardiovasc. Pharmacol. 58, 413–459.

Chidiac, P., Hebert, T.E., Valiquette, M., Dennis, M., Bouvier, M., 1993. Inverse agonist activity of b-adrenergic antagonists. Mol. Pharmacol. 45, 490–499.

Chuang, T.T., Lacovelli, L., Sallese, M., DeBlasi, A., 1996. G-protein-coupled receptors: heterologous and homologous desensitization and its implications. TiPS 17, 416–421.

Chung, K.Y., Rasmussen, S.G., Liu, T., Li, S., DeVree, B.T., Chae, P.S., Calinski, D., Kobilka, B.K., Woods, V.L., Sunahara, R.K., 2011. Conformational changes in the G-protein Gs induced by the β2 adrenergic receptor. Nature 477, 611–615.

Clapham, D.E., Neer, E.J., 1997. G protein βγ subunits. Ann. Rev. Pharm. Tox. 37, 167–203.

Craven, K.B., Zagotta, W.N., 2006. CNG and HCN channels: two peas, one pod. Ann. Rev. Physiol. 68, 375–401.

Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H., Kirk, C.J., 1983. Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones. Biochem. J. 212, 733–747.

Darmon, M., Brachet, P., Pereira da Silva, L.H., 1975. Chemotactic signals induce cell differentiation in Dictyostelium discoideum. PNAS U.S.A. 72, 3163–3166.

Diel, S., Beyermann, M., Llorens, J.M.N., Wittig, B., Kleusst, C., 2008. Two interaction sites on mammalian adenylyl cyclase type I and II: modulation by calmodulin and Gβγ. Biochem. J. 411, 449–456.

Dixon, R.A., et al., 1986. Cloning of the gene and cDNA for mammalian b-adrenergic receptor and homology with rhodopsin. Nature 321, 75–79.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L.O., Dohlman, H.G., Friele, T., et al., 1986. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature 321, 75–79.

Dror, R.O., Arlow, D.H., Borhani, D.W., Jensen, M.O., Piana, S., Shaw, D.E., 2009. Identification of two distinct inactive conformations of the β2-adrenergic receptor reconciles structural and biochemical observations. PNAS U.S.A. 106, 4689–4694.

Ehlert, F.J., 1986. 'Inverse agonists,' cooperativity and drug action at benzodiazepine receptors. Trends Pharmacol. Sci. 7, 28–32.

Eto, M., Kiazawa, T., Matsuzawa, F., Aikawa, D.-I., Kirkbride, J.A., Isozumi, N., Nishimura, Y., Brautigan, D.L., Ohki, S.-Y., 2007. Phosphorylation-induced conformational switching of CPI-17 produces a potent myosin phosphatase inhibitor. Structure 15, 1591–1602.

Fischmeister, R., Castro, L.R.V., Abi-Gerges, A., Rochais, F., Jurevicius, J., Leroy, J., Vandecasteele, G., 2006. Compartmentation of cyclic nucleotide signalling in the heart. Cir. Res. 99, 816–828.

Franchini, K.G., 2012. Focal adhesion kinase – the basis of local hypertrophic signalling domains. J. Mol. Cell Cardiol. 52, 485–492.

Frisch, S.M., Vuori, K., Ruoslahti, E., Chan-Hui, P.Y., 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. 134, 793–799.

Fu, X., Koller, S., Abd Alla, J., Quitterer, U., 2013. Inhibition of G-protein-coupled receptor kinase 2 (GRK2) triggers the growth-promoting mitogen-activated protein kinase (MAPK) pathway. J. Biol. Chem. 288, 7738–7755.

Galandrin, S., Oligny-Longpre, G., Bonin, H., Ogawa, K., Gales, C., Bouvier, M., 2008. Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the β1-adrenergic receptor. Mol. Pharmacol. 74, 162–172.

Gancedo, J.M., 2013. Biological roles of cAMP: variations on a theme in the different kingdoms of life. Biol. Rev. 88, 645–668.

Gloerich, M., Bos, J.L., 2010. Epac: defining a new mechanism for cAMP action. Ann. Rev. Pharmacol. Toxicol. 50, 355–375.

Goody, R.S., 2003. The missing link in the muscle cross-bridge cycle. Nat. Struct. Biol. 10, 773–775.

Gorke, B., Stulke, J., 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat. Rev. Microbiol. 6, 613–624.

Grant, A., Lee, D., Orengo, C., 2004. Progress towards mapping the universe of protein folds. Genome Biol. 5, 107.

Gresset, A., Sondek, J., Harden, T.K., 2012. The phospholipase C isozymes and their regulation. Subcell. Biochem. 58, 61–94.

Harvey, P.A., Leinwand, L.A., 2011. Cellular mechanisms of cardiomyopathy. J. Cell Biol. 194, 355–365.

Hokin, M.R., Hokin, L.E., 1953. Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J. Biol. Chem. 203, 967–977.

Huang, C.-C., Tesmer, J.J.G., 2011. Kinases with activated GPCRs and G protein-coupled receptor (GPCR) interactions of heterotrimeric G proteins recognition in the face of diversity. J. Biol. Chem. 286, 7715–7721.

Hurley, J.H., 1999. Structure, mechanism, and regulation of mammalian adenylyl cyclase. J. Biol. Chem. 274, 7599–7602.

Huxley, H.E., Hanson, J., 1954. Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature 173, 973–997.

Huxley, A.F., Niedergerke, R., 1954. Structural changes in muscle during contraction: interference microscopy of living muscle fibres. Nature 173, 971–973.

Kahsai, A.W., Xiao, K., Rajagopal, S., Ahn, S., Shukla, A.K., Sun, J., Oas, T.G., Lefkowitz, R.J., 2011. Multiple ligand-specific conformations of the β2-adrenergic receptor. Nat. Chem. Biol. 7, 692–700.

Kang, D.S., Kern, R.C., Puthenveedu, M.A., von Zastrow, M., Williams, J.C., Benovic, J.L., 2009. Structure of an arrestin2-clathrin complex reveals a novel clathrin binding domain that modulates receptor trafficking. J. Biol. Chem. 284, 29860–29872.

Kay, R.R., Flatman, P., Thompson, C.R., 1999. DIF signalling and cell fate. Semin. Cell Dev. Biol. 10, 577–585.

Kelly, E., 2013. Efficacy and ligand bias at the μ-opioid receptor. Br. J. Pharmacol. 169, 1430–1446.

Kim, Y.-M., Benovic, J.L., 2002. Differential roles of arrestin-2 interaction with clathrin and adaptor protein-2 in G protein-coupled receptor trafficking. J. Biol. Chem. 277, 30760–30768.

Kovacs, J.J., Whalen, E.J., Liu, R., et al., 2008. β-arrestin mediated localization of smoothened to the primary cilium. Science 320, 1777–1781.

Kraetler, M.J., Solits, A.R., Saucerman, J.J., 2010. Modelling cardiac β-adrenergic signalling with normalized-Hill differential equations: comparison with a biochemical model. BMC Syst. Biol. 4, 157.

Krupinski, J., Coussen, H.A., Bakalyar, H.A., Tang, W.-J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R., Gilman, A.G., 1989. Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science 244, 1558–1564.

Kuhn, H., 1978. Light regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membrane. Biochemistry 17, 4389–4395.

Kull, F.J., Endow, S.A., 2013. Force generation by kinesin and myosin cytoskeletal motor proteins. J. Cell Sci. 126, 9–19.

Laporte, S.A., Oakley, R.H., Holt, J.A., Barak, L.S., Caron, M.G., 2000. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. J. Biol. Chem. 275, 23120–23126.

Larsson, H.P., 2010. How is the heart rate regulated in the sinoatrial node? another piece to the puzzle. J. Gen. Physiol. 136, 237–241.

Lefkowitz, R.J., 2013. Arrestins come of age: a personal historical perspective. Progess Mol. Biol. Transl. Sci. 118, 3–17.

Leightling, B.H., Rickenberg, H.V., Seely, R.J., Fahrney, D.E., Pace, N.R., 1986. The occurrence of cyclic AMP in archaebacteria. Biochem. Biophys. Res. Commun. 136, 1078–1082.

Li, W.W., Luan, S., Schreiber, S.L., Assmann, S.M., 1994. Cyclic AMP stimulates K^+ channel activity in mesophyll cells of Vicia faba. Plant Physiol. 105, 957–961.

Linder, J.U., Schultz, J.E., 2003. The class III adenylyl cyclases: multi-purpose signalling modules. Cell Signal 15, 1081–1089.

Linder, J.U., Schultz, J.E., 2008. Versatility of signal transduction encoded in dimeric adenylyl cyclases. Curr. Opin. Struct. Biol. 18, 667–672.

Liu, J.J., Horst, R., Katritch, V., Stevens, R.C., Wuthrich, K., 2012. Biased signalling pathways in β2-adrenergic receptors characterized by 19F-NMR. Science 335, 1106–1110.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., Lefkowitz, R.J., 1990. β-Arrestin: a protein that regulates β-adrenergic receptor function. Science 248, 1547–1550.

Luttrell, L., Miller, W.E., 2013. Arrestins as regulators of kinases and phosphatases. Prog. Mol. Biol. Transl. Sci. 118, 115–147.

Luttrell, L.M., Ferguson, S.S.G., Kaaka, Y., et al., 1999. β-arrestin-dependent formation of β2-adrenergic receptor/Src kinase complexes and mitogenic signalling. Science 283, 655–661.

MacLennan, D.H., Kranias, E.G., 2003. Phospholamban: a crucial regulator of cardiac contractility. Nat. Rev. Mol. Cell Biol. 4, 566–577.

Maron, B.J., Towbin, J.A., Thiene, G., Antzelvitch, C., Corrado, D., et al., 2006. Contemporary definitions and classification of cardiomyopathies. Circulation 113, 1807–1816.

Menard, L., Ferguson, S.G., Barak, L.S., et al., 1996. Members of the G protein-coupled receptor kinase family that phosphorylate the β2-adrenergic receptor facilitate sequestration. Biochemistry 35, 4155–4160.

Michell, R.H., 1975. Inositol phospholipids in cell surface receptor function. Biochim. Biophys. Acta 415, 81–147.

Morris, A.J., Malbon, C.C., 1999. Physiological regulation of G protein-linked signaling. Physiol. Rev. 79, 1373–1430.

Mou, T.-C., Masada, N., Cooper, D.M.F., Sprang, S.R., 2009. Structural basis for inhibition of mammalian adenylyl cyclase by calcium. Biochem 48, 3387–3397.

Nakamura, N.T., Tomomura, A., Kato, S., Noda, C., Ichihara, A., 1984. Reciprocal expressions of a_1- and b-adrenergic receptors, but constant expression of glucagon receptor by rat hepatocytes during development and primary culture. J-Biochem-Tokyo 96, 127–136.

Nikolaev, V.O., Moshkov, A., Lyon, A.R., Miragoli, M., Novak, P., Paur, H., Lohse, M.J., Korchev, Y.E., Harding, S.E., Gorelik, J., 2010. β2-adrenergic receptor distribution in heart failure changes cAMP compartmentation. Science 327, 1653–1657.

Nishizuka, Y., 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258, 607–614.

Nobles, K.N., Xiao, K., Ahn, S., Shukla, A.K., Lam, C.M., et al., 2011. Distinct phosphorylation sites on the β2-adrenergic receptor establishes a barcode that encodes differential functions of β-arrestin. Sci. Signal 4 (185), ra51.

Noma, T., Lemaire, A., Naga Prasad, S.V., Barki-Harrington, L., Tilley, D.G., Chen, J., et al., 2007. beta-Arrestin-mediated beta(1)-adrenergic receptor transactivation of the EGFR confers cardioprotection. J. Clin. Invest 117, 2445–2458.

Nygaard, R., Zou, Y., Dror, R.O., Mildorf, T.J., Arlow, D.H., et al., 2013. The dynamic process of β2-adrenergic receptor activation. Cell 152, 532–542.

Ohki, S.-Y., Eto, M., Kariya, E., Hayano, T., Hayashi, Y., Yazawa, M., Brautigan, D., Kainosho, M., 2001. Solution NMR structure of the myosin phosphatase inhibitor CPI-17 shows phosphorylation-induced conformational changes responsible for activation. J. Mol. Biol. 314, 839–849.

Palmitter, R.D., 2007. Is dopamine a physiological relevant mediator of feeding behavior? Trends Neurosci. 30, p375–381.

Pentassuglia, L., Sawyer, D.B., 2012. ErbB/integrin signalling interactions in regulation of myocardial cell-cell and cell-matrix interactions. Biochim. Biophys. Acta 1833, 909–916.

Pentassuglia, L., Sawyer, D.B., 2013. Erb/integrin signalling interactions in regulation of myocardial cell-cell and cell-matrix interactions. Biochim. Biophys. Acta Mol. Cell Res. 1833, 909–916.

Pfister, C., Chabre, M., Plouet, J., Tuyen, V.V., DeKozak, Y., Faure, J.P., Kuhn, H., 1985. Retinal S antigen identified as the 48 kDa protein regulating light-dependent phosphodiesterase in rods. Science 228, 891–893.

Postea, O., Biel, M., 2011. Exploring HCN channels as novel drug targets. Nat. Rev. Drug Discov. 10, 903–914.

Prenzel, N., Zwick, E., Daub, H., Lesener, M., Abraham, R., Wallasch, C., Ullrich, A., 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402, 884–888.

Pyle, W.G., Solaro, R.J., 2004. At the crossroads of myocardial signalling: the role of Z-discs in intracellular signalling and cardiac function. Circ. Res. 94, 296–305.

Rall, T.W., Sutherland, E.W., Berthet, J., 1957. The relationship of epinephrine and glucagon to liver phosphorylase: effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. J. Biol. Chem. 224, 463–475.

Rang, H.P., Dale, M.M., Ritter, J.M., Flower, R.J., 2008. Pharmacology, sixth ed. Churchill Livingstone Elsevier.

Rasmussen, S.G.F., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., et al., 2011. Crystal structure of the β2 adrenergic receptor-GS protein complex. Nature 477, 549–555.

Rayment, I., Holden, H.M., Whittaker, M., Yohn, C.B., Lorenz, M., Holmes, K.C., Milligan, R.A., 1993. Structure of the actin-myosin complex and its implications for muscle contraction. Science 261, 58–65.

Rosenbaum, D.M., Zhang, C., Lyons, J.A., Hool, R., Aragao, D., Arlow, D., Rasmussen, S.G.F., Choi, H.-J., DeVree, B.T., Sunahara, R.K., Chae, P.S., Gellman, S.H., Dror, R.O., Shaw, D.E., Weis, W.I., Caffrey, M., Gmeiner, P., Kobilka, B.K., 2011. Structure and function of an irreversible agonist-β2 adrenoceptor complex. Nature 469, 236–240.

Ross, E.M., 2008. Coordinating speed and amplitude in G-protein signaling. Curr. Biol. 18, R777–R783.

Rubin, R.P., 2007. A brief history of great discoveries in Pharmacology. In celebration of the centennial anniversary of the founding of the American Society of Pharmacology and Experimental Therapeutics. Pharmacol. Rev. 59, 289–359.

Scheerer, P., Park, J.H., Hildebrand, P.W., Kom, Y.J., Krauss, N., Choe, H.W., Homann, K.P., Ernst, O.P., 2008. Crystal structure of the active G-protein-coupled receptor opsin in complex with a C-terminal peptide derived from the Galpha subunit of transducin. Nature 455, 497–502.

Seo, M.-D., Velamakanni, S., Ishiyama, N., Sathopulos, P.B., Rossi, A.M., Khan, S.A., Dale, P., Li, C., Ames, J.B., Ikura, M., Taylor, C.W., 2010. Structural and functional conservation of key domains in InsP3 and ryanodine receptors. Nature 483, 108–112.

Shinohara, T., Dietzschold, B., Craft, C.M., et al., 1987. Primary and secondary structure of bovine retinal S antigen (48-kDa protein). PNAS U.S.A. 84, 6975–6979.

Shukla, A.K., Xiao, K., Lefkowitz, R.J., 2011. Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signalling. Trends Bioch. Sci. 36, 457–469.

Shukla, A.K., Manglik, A., Kruse, A.C., Xiao, K., Reis, R.I., et al., 2013. Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. Nature 497, 137–142.

Southwick, S.M., Bremner, J.D., Rasmusson, A., Morgan, C.A., Arnsten, A., Charney, D.S., 1999. Role of norepinephrine in the pathophysiology and treatment of posttraumatic stress disorder. Biol. Psychiatry 46, 1192–1204.

Starling, E.H., 1923. The Wisdom of the Body. Brit. Med. J. 2, 685–690.

Streb, H., Irvine, R.F., Berridge, M.J., Schultz, I., 1983. Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306, 67–69.

Sutherland, E.W., Rall, T.W., Menon, T., 1962. Adenylyl cyclase: distribution, preparation and properties. J. Biol. Chem. 237, 1220–1227.

Sutherland, E.W., 1972. Studies on the mechanism of hormone action. Science 177, 401–408.

Tang, W.-J., Guo, Q., 2009. The adenylyl cyclase activity of anthrax edema factor. Mol. Asp. Med. 30, 423–430.

Taylor, C.W., da Fonseca, P.C.A., Morris, E.P., 2004. IP3 receptors: the search for structure. TiBS 29, 210–219.

Tesmer, J.J., Berman, D.M., Gilman, A.G., Sprang, S.R., 1997a. Structure of RGS4 bound to Al F4-activated Gαi1: stabilization of the transition state for GTP hydrolysis. Cell 89, 251–261.

Tesmer, J.J., Sunahara, R.K., Gilman, A.G., Sprang, S.R., 1997b. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsalpha.GTPgammaS. Science 278, 1907–1916.

Tesmer, J.J., Dessauer, C.W., Sunahara, R.K., Murray, L.D., Johnson, R.A., Gilman, A.G., Sprang, S.R., 2000. Molecular basis for P-site inhibition of adenylyl cyclase. Biochemistry 39, 14464–14471.

Thevelein, J.M., de Winde, J.H., 1999. Novel sensing mechanism and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 33, 904–918.

Todaka, K., Ogino, K., Gu, A., Burkhoff, D., 1998. Effect of ventricular stretch on contractile strength, calcium transient and cAMP in intact canine hearts. Am. J. Physiol. 274, H990–H1000.

Tresguerres, M., Levin, L.R., Buck, J., 2011. Intracellular cAMP signaling by soluble adenylyl cyclase. Kidney Int. 79, 1277–1288.

Unglaub-Silverthorn, D., 2010. Human Physiology; An Integrated Approach, fifth ed. Pearson/Benjamin Cummings, San Francisco.

Usher, M., Cohen, J.D., Servan-Schreiber, D., Rajkowski, J., Aston-Jones, G., 1999. The role of locus coeruleus in the regulation of cognitive performance. Science 283, 549–554.

Vasudevan, N.T., Mohan, M.L., Gupta, M.K., Hussain, A.K., Naga Prasad, S.V., 2011. Inhibition of protein phosphatase 2A activity by PI3Kγ regulates β-adrenergic receptor function. Mol. Cell 41, 636–648.

Vasudevan, N.T., Mohan, M.L., Gupta, M.K., Martelli, E.E., Hussain, A.K., Qin, Y., Chandrasekharan, U.M., Young, D., Feldman, A.M., Sen, S., Dorn, G.W., Dicorleto, P.E., Naga Prasad, S.V., 2013. Gβγ-independent recruitment of G-protein coupled receptor kinase 2 drives tumor necrosis factor α-induced cardiac β-adrenergic receptor dysfunction. Circulation 128, 377–387.

Vostrikov, V.V., Mote, K.R., Verardi, R., Veglia, G., 2013. Structural dynamics and topology of phosphorylated phospholamban homopentamer reveals its role in the regulation of calcium transport. Structure 21, 1–12.

Waldo, G.L., Ricks, T.K., Hicks, S.N., Cheever, M.L., Kawano, T., Tsuboi, K., Wang, X., Montell, C., Kozasa, T., Sondek, J., Harden, T.K., 2010. Kinetic scaffolding mediated by a phospholipase C-β and Gq signaling complex. Science 330, 974–980.

Warne, T., Moukhametzianov, R., Baker, J.G., Hehmé, R., Edwards, P.C., Leslie, A.G.W., Schertler, F.X., Tate, C.G., 2011. The structural basis for agonist and partial agonist action on a β1-adrenergic receptor. Nature 469, 241–244.

Welch, E.J., Jones, B.W., Scott, J.D., 2010. Networking with AKAPs: context-dependent regulation of anchored enzymes. Mol. Interv. 10, 86–89.

Westfield, G.H., Rasmussen, S.G.F., Su, M., Dutta, S., De Vree, B.T., Chung, K.Y., et al., 2011. Structural flexibility of the Gαs-helical domain in the β2-adrenoceptor Gs complex. PNAS U.S.A. 108, 16086–16091.

Whorton, M.R., McKinnon, R., 2013. X-ray structure of the mammalian GIRK2-βγ G-protein complex. Nature 498, 190–197.

Wilden, U., Hall, S.W., Kühn, H., 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. PNAS U.S.A. 83, 1174–1178.

Wisler, J.W., DeWire, S.M., Whalen, E.J., Violin, J.D., Drake, M., Ahn, S., Shenoy, S.K., Lefkowitz, R.J., 2007. A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. PNAS U.S.A. 104, 16657–16662.

Xiao, K., McClatchy, D.B., Shukla, A.K., et al., 2007. Functional specialization of b-arrestin interactions revealed by proteomic analysis. PNAS U.S.A. 104, 12011–12016.

Xiao, K., Sun, J., Kim, J., Rajagopal, S., Zhai, B., Villen, J., Haas, W., et al., 2010. Global phosphorylation analysis of β-arrestin-mediated signalling downstream of a seven transmembrane receptor (7TMR). PNAS 107, 15299–15304.

Zaugg, M., Xu, W., Lucchinetti, E., Shafiq, S.A., Jamali, N.Z., Siddiqui, M.A., 2000. Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. Circulation 102, 344–350.

Zhang, G., Liu, Y., Ruoho, A.E., Hurley, J.H., 1997. Structure of the adenylyl cyclase catalytic core. Nature 386, 247–253.

Zhang, L., Malik, S., Kelley, G.G., Kapiloff, M.S., Smrcka, A.V., 2011. Phospholipase C epsilon scaffolds to muscle-specific A kinase anchoring protein (mAKAPbeta) and integrates multiple hypertrophic stimuli in cardiac myocytes. J. Biol. Chem. 286, 23012–23021.

Zhu, W.Z., Zheng, M., Koch, W.J., Lefkowitz, R.J., Kobilka, B.K., Xiao, R.P., 2001. Dual modulation of cell survival and cell death by β2-adrenergic signaling in adult mouse cardiac myocytes. Proc. Natl. Acad. Sci. U.S.A. 98, 1607–1612.

Zocher, M., Fung, J.J., Kobilka, B.K., Muller, D.J., 2012. Ligand-specific interactions modulate kinetic, energetic and mechanical properties of the human β2 adrenergic receptor. Structure 20, 1391–1402.

CHAPTER

4

Cholinergic Signaling and Muscle Contraction

> The next night, at three o'clock, the idea returned. It was the design for an experiment to determine whether or not the hypothesis of a chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart according to the nocturnal design.
> ***Otto Loewi***

ACETYLCHOLINE

Acetylcholine was the first neurotransmitter to be identified. Henry Hallett Dale recognized it as a contaminant from Ergot and described in 1914 its action as both "muscarinic" and "nicotinic." A few years later, Otto Loewi confirmed that it acted as a neurotransmitter, which he initially named "vagusstoff," a substance released by electric stimulation of the vagus nerve and which causes a reduction in the heart rate (Loewi, 1921). It operates in the autonomic nervous system, acting both centrally and peripherally and it is the only neurotransmitter used in the motor division of the somatic nervous system (motor neurons of skeletal muscles). Important anatomical loci in the brain are the nucleus of Meynert, the amygdala, the hippocampus, and numerous brain stem nuclei. Its release plays a role in cognitive functions, especially memory, as well as stimulating goal-directed behavior (attention). The gradual loss of cholinergic neurons that connect the hippocampus with the entorhinal cortex, lies at the start of a progressive degenerative process that ends with Alzheimer's disease (Hernandez and Dineley, 2012). In the periphery, all ganglia of the sympathetic and parasympathetic system operate through acetylcholine (return to Chapter 3, Figure 3-2). Acetylcholine causes fluid secretion from the pancreas, salivary, and sweat glands. It also causes vasodilatation but as we learn in this chapter, this is not a direct effect on smooth muscle; it is the consequence of the generation of nitric oxide. Acetylcholine is synthesized in the cytoplasm

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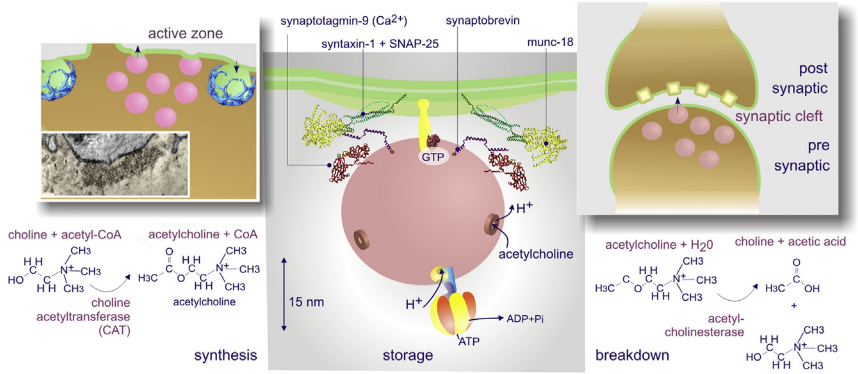


FIGURE 4-1 Production and breakdown of acetylcholine. Acetylcholine is produced in the cytoplasm by choline acetyltransferase and loaded into secretory vesicles with the help of an H^+/acetylcholine cotransporter (antiporter). The necessary proton gradient is brought into place by a proton pump (H^+-ATPase). These vesicles attach to the plasma membrane at the "active zone." They contain a SNARE protein (synaptobrevin) and a Ca^{2+}-binding protein (synaptotagmin-9). Complementary SNARES are projected from the plasma membrane (syntaxin-1 and SNAP-25). Upon a secretory signal, Ca^{2+} influx, the vesicle fuses with the membrane and releases its content into the synaptic cleft (numerous vesicles at the time). The signal is propagated by binding of acetylcholine to its receptor on the postsynaptic membrane. Acetylcholine esterase assures a rapid removal of acetylcholine, giving rise to only a very brief signal (unless new acetylcholine is released into the cleft). Clostridial neurotoxins damage the SNARE proteins and prevent membrane fusion.

of nerves by choline acetyltransferase, starting from choline and acetyl CoA. It accumulates in synaptic vesicles thanks to a proton-driven cotransporter.

In the example of neuromuscular transmission, the released acetylcholine diffuses across the junctional cleft, which contains the basement membrane surrounding the muscular cell, and interacts with receptors on the postsynaptic membrane. It is then removed with "flashlike suddenness" (in the words of Henry Hallett Dale). This occurs in part by diffusion, but mainly by the action of acetylcholinesterase, which breaks it down, within milliseconds, into choline and acetate (Figure 4-1). Since the affinity for receptors in the neuromuscular junction is rather moderate (you need a μM concentration to get sufficient receptor occupation, compared to growth factors that operate in the nM range), the rate of dissociation is sufficiently fast to allow acetylcholine to rapidly detach following the steep decline in the local concentration. This scenario is ideal for rapid transient transmission as occurs, for instance, during skeletal muscle contraction when you are running. Because of the extreme lability of acetylcholine, in experimental investigations it has been normal to work with stable nonhydrolyzable derivatives such as carbamylcholine (carbachol).

BOTULINUM NEUROTOXIN

The release of acetylcholine can be blocked by botulinum which is a neurotoxin produced by *Clostridium botulinum* (Gram-positive rod-shaped bacterium). The toxin is a big protein that enters synaptic endings of motor neurons (cholinergic nerve terminals) where they bind members of the SNARE family such as synaptobrevin, SNAP-25 and syntaxin-1, and cause their cleavage. The SNARE proteins are instrumental in the fusion of secretion vesicles with the outer membrane and their loss of function prevents acetylcholine release. The immediate clinical problem of patients poisoned by *Clostridium botulinum* is flaccid paralysis and respiratory failure. In humans, the bacteria enter the body in the form of spores through badly preserved food (canned food or smoked meat) (Binz et al., 2010).

MASKS OF PERFECTION

Botulinum toxin A is used in cosmetic surgery, the so-called Botox treatment, often in combination with "fillers" such as collagen. The toxin is injected under the skin and inhibits muscles whose contraction causes wrinkles (frown lines). The effect sets in after two weeks, giving rise to a smooth forehead, and lasts for up to six months. http://marcerwinbabej.com/portfolio/

ACETYLCHOLINESTERASE INHIBITORS

Some of the compounds that inhibit the hydrolysis of acetylcholine are among the most toxic known. Thus, they initially cause fasciculations (muscle twitches), myotonic jerks and, as a consequence of ganglionic stimulation of the adrenal gland, they cause hyperglycemia, hypertension, mydriasis (papillary dilatation), sweating, tachycardia (quick heartbeat), and tachydysrhythmias. This phase is followed by muscle weakness and paralysis because the prolonged presence of acetylcholine causes irreversible acetylcholine receptor desensitization. Organophosphorus compounds are among the best known acetylcholinesterase inhibitors. They were developed with the aim of preparing nasty surprises for flies (Parathion, the most widely used insecticide of this class), weeds (Roundup) and subsequently even nastier surprises for soldiers and civilians too. These "nerve gases" are among the list as weapons of mass destruction. Among these, Sarin was used to eliminate civilians by Saddam Hussein's forces in 1988, by zealots

continued

ACETYLCHOLINESTERASE INHIBITORS *(cont'd)*

of the Aum cult in Tokyo in 1995 and by Syrian soldiers in 2013. The Nobel Peace Prize of 2013 was awarded to the Organization for the Prohibition of Chemical Weapons "for its extensive efforts to eliminate chemical weapons." Atropine, a muscarinic receptor antagonist, is used as antidote. Casualties are also treated with Pralidoxime, with the aim of dephosphorylating and so reactivating the poisoned acetylcholinesterase (Sellin and McArdle, 1994; van Helden et al., 1996). Some cholinesterase inhibitors are less toxic, because of a more reversible action, and have found their way into clinical practice as in the treatment of myasthenia gravis (Tensilin, Prostigmin, Mestinon) and Alzheimer's disease (Aricept, Exelon, and Reminyl).

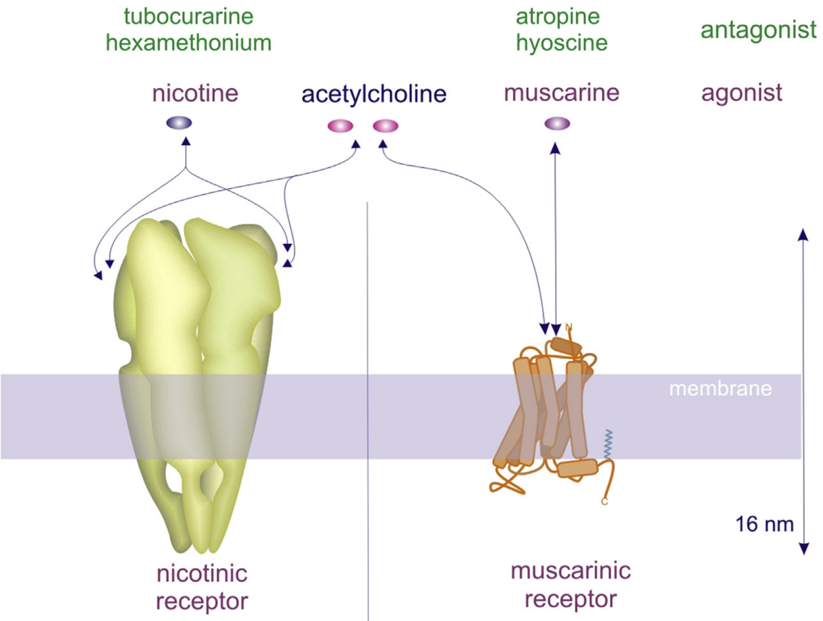
CHOLINERGIC RECEPTOR SUBTYPES; NICOTINIC AND MUSCARINIC

Acetylcholine receptors fall into two classes, originally distinguished, much the same way as for adrenoceptors, by the employment of pharmacological agonists and antagonists. Thus, it was shown by John Langley that the action of nicotine causes tonic contraction of certain muscles of fowl and frogs, and that this was prevented by curare (Langley, 1907). Henry Dale distinguished the actions of muscarine and nicotine, leading to the recognition of two pharmacological distinct families of acetylcholine receptors that take their names from these natural products (Dale, 1914). The receptor activated by nicotine was named nicotinic acetylcholine receptor (nAChR) and tubocurarine is its prototypic antagonist, whereas the one activated by muscarine was named muscarinic acetylcholine receptor (mAChR) and it is blocked by atropine. Remember, acetylcholine binds both types of receptors, albeit it with much greater affinity for muscarinic (K_D of ~30 nM), than for nicotinic (K_D of ~30 µM for muscular). Unlike the adrenaline receptors, the pharmacological distinct types correspond to structurally and functionally totally unrelated proteins (Figure 4-2).

Web resource of cholinergic receptors http://www.genenames.org/genefamilies/CHR.

NICOTINIC ACETYLCHOLINE RECEPTORS

The nicotinic acetylcholine receptors constitute a family of pentameric (five subunits) cation channel which conducts Na^+, K^+, and to a variable extent Ca^{2+} and so are termed ionotropic receptors. These channels are sufficiently



membrane depolarization (or Ca^{2+} entry) "ionotropic"	G-protein regulated effectors "metabotropic"	
Nmuscular -neuromuscular junction Nneuron -brain -peripheral nervous system -chromaffin cells (adrenal medulla)	M1	-brain, -autonomic ganglia -glands
	M2	-heart -brain
	M3	-smooth muscle (airway, gut, bladder, eye) -glands (salivary, gastric, sweat, airway submucosal) -endothelium -brain
	M4	-brain
	M5	-brain (substantia nigra -salivary gland -smooth muscle eye

FIGURE 4-2 Two types of acetylcholine receptors with radically different signaling properties. The nicotinic receptor is a ligand-gated ion channel, whereas the muscarinic receptor is a G-protein-coupled protein. Nicotinic acetylcholine receptors are composed of five proteins (a pentamer) which exist in different forms, either constituting muscular or neuronal channels. Muscarinic acetylcholine receptors exist in five different versions, some broadly others more specifically expressed.

wide to allow water to accompany the flow of ions and, as a consequence, are necessarily less ion-selective (unlike, for instance, the highly selective potassium channels (Doyle et al., 1998)). The receptor and ion channel are intrinsic properties of the same protein. Based on sequence analysis and structural organization, they qualify as members of the "cys-loop" superfamily of channels that include (in humans) receptors for 5-hydroxytryptamine (HTR3), glycine (GLRA), and γ-aminobutyric acid (GABA). Nicotinic acetylcholine receptors are expressed at postsynaptic membranes of neuromuscular junctions and of synapses in the autonomic ganglions. They occur on chromaffin cells in the adrenal medulla (return to Chapter 3, Figure 3-2) and they occur at pre-, post-, and perisynaptic membranes in the central nervous system (where some of these receptors exert the (addictive) psychoactive actions of nicotine in tobacco smoke). However, nicotinic acetylcholine receptors are also expressed in nonexcitable tissues such as skin keratinocytes, vascular endothelial cells, lung epithelial cells, cytotoxic T-lymphocytes, and a subpopulation of hematopoietic progenitor cells, among others (Albuquerque et al., 2009). We will focus on signaling downstream of the nicotinic acetylcholine receptor at the neuromuscular junction.

To date, 17 human nAChR subunits have been identified. They are classified into four types (I–IV) based on similarities in protein sequence (Lukas et al., 1999). All nicotinic receptors contain one or more α subunits, generally combined with a variety of other subunits giving rise to heteropentamers. As an exception, α7 and α9 subunits can exist as homopentamers (this also applies to a chicken-specific α8 subunit). Subunit composition is difficult to assert and for numerous receptor combinations the "wild card" is still employed, in which unknown subunits are indicated by an asterisk (examples, α7*-nAChR or α4β2*-nAChR) (Figure 4-3). Binding of the ligand occurs at the interface between two subunits: one face offering a "principal side," the other "a complementary side." Observed interfaces are α-α, α-β, and, in the case of the muscular receptor, α1-δ. As a consequence, the number of binding sites varies per given subunit combination.

NICOTINE, MUSCARINE, AND ATROPINE

- Nicotine was named after Jacques Nicot, the French ambassador at Lisbon who introduced the tobacco plant into France in 1560. Nicotine is produced by plants as a defense to predation by insects, it is a natural pesticide.
- Muscarine is a product of the poisonous mushroom, *Amanita muscaria*.
- Atropine is an alkaloid extracted from berries of the deadly nightshade "*Atropa belladonna*." The plant was so named because of the lethal consequences of eating its fruits, hence *Atropa* referring to the Greek *a-tropos*, meaning "no turning" or "inflexible" and because the extract was used in "cosmetic eye droplets" to dilate the pupil, hence belladonna or "beautiful woman." It acts as a nonselective muscarinic acetylcholine receptor antagonist.

SMOKERS AND NICOTINIC RECEPTORS

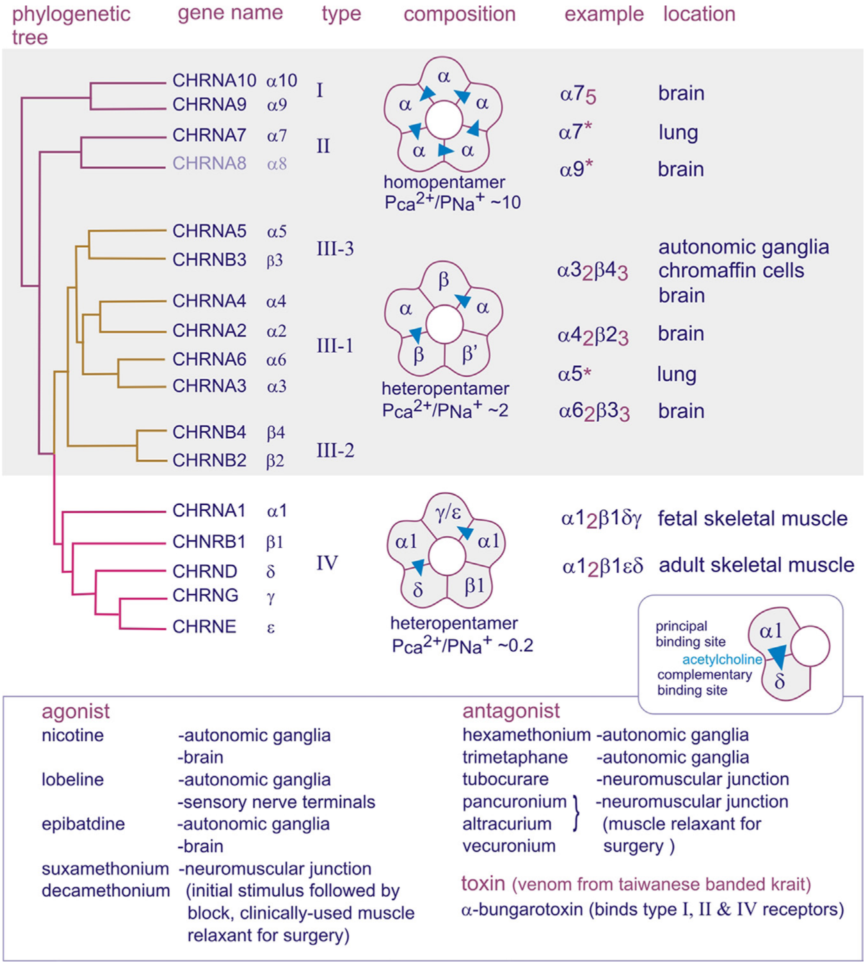
α4β2-containing nicotinic acetylcholine receptors (proposed stoichiometry of $\alpha 4_2 \beta 2_3$) have an unusual high affinity for nicotine (K_D below 1 μM) and they account for >90% of radiolabeled ^{3}H-nicotine binding to brain tissues. Transgenic knockout of either of these subunits eliminates nicotine self-administration in mice, whereas ectopic expression recovers this behavior. These findings imply that α4β2 plays a role in nicotine addiction. Nicotine administration provides both reward signals (connected with dopamine neurons) and anxiety relief, actions that seem to require α4β2 receptors (McGranahan et al., 2011). Nicotinic acetylcholine receptors are also expressed in lungs. Expression of α5, α7, and β4 has been detected but the exact combination of subunits has not been revealed (Maus et al., 1998). Single nucleotide polymorphism (snp) of these genes was found to be associated with the incidence of lung cancer (Hung et al., 2008). This finding suggests that, besides the classic carcinogenic components of tobacco smoke, such as polycyclic aromatic hydrocarbons and methylnitrosamino-pyridyl-butanone, nicotine itself may play a role in cancer development (Hecht, 1999).

TUBOCURARINE

It is the main constituent of curare, a mixture of plant alkaloids obtained from *Chondrodendron tomentosum* or *Strychnos toxifera*, used as an arrow poison by some South American Indians. Claude Bernard (1856) showed that it causes paralysis by blocking neuromuscular transmission. Langley (in 1905) suggested that it acts by combining with the receptive substance at the motor end plate (see Chapter 1, Prologue). Other related (synthetic) compounds, such as gallamine differ mainly in their duration of action (Bernard, 1956; Langley, 1905).

The nicotinic acetylcholine receptor at the neuromuscular junction; electric organs provide a source for research

Motor neurons terminating on skeletal muscles form a structure, the neuromuscular junction or end plate, that is particularly rich in nicotinic acetylcholine receptors, attaining 40 million copies on the muscle outer membrane (sarcolemma) (Figure 4-4). Because it is difficult to grow crystals of entire transmembrane proteins, the three-dimensional



agonist	
nicotine	-autonomic ganglia -brain
lobeline	-autonomic ganglia -sensory nerve terminals
epibatdine	-autonomic ganglia -brain
suxamethonium decamethonium	-neuromuscular junction (initial stimulus followed by block, clinically-used muscle relaxant for surgery)

antagonist	
hexamethonium	-autonomic ganglia
trimetaphane	-autonomic ganglia
tubocurare	-neuromuscular junction
pancuronium altracurium vecuronium	-neuromuscular junction (muscle relaxant for surgery)

toxin (venom from taiwanese banded krait)

α-bungarotoxin (binds type I, II & IV receptors)

FIGURE 4-3 Different subunit combinations of nicotinic acetylcholine receptors and their main sites of expression. Note the clear distinction between the composition of neuronal (gray section) and muscle receptors. The blue arrows depict acetylcholine, with five binding sites for monopentameric receptors and two binding sites for heteropentameric. An asterisk indicates that accompanying subunits are not determined. With respect to the cations, the types I and II are predominantly Ca^{2+} channels (permeability $Ca^{2+} > Na^+$), for type III the permeability for Ca^{2+} and Na^+ is nearly identical, whereas type IV receptors mainly transports Na^+ (mainly inward currents under physiological conditions). All channels K^+ are permeable for K^+ (mainly outward current under physiological conditions). The list of agonists and antagonists shown at the bottom of the image is not exhaustive.

structure of the nicotinic receptor has initially been studied by cryoelectron microscopy prepared from the receptor-rich postsynaptic membranes of the electromotor system of *Torpedo marmorata* (Brisson and Unwin, 1984; Miyazawa et al., 2003). The electromotor system, or electric organ,

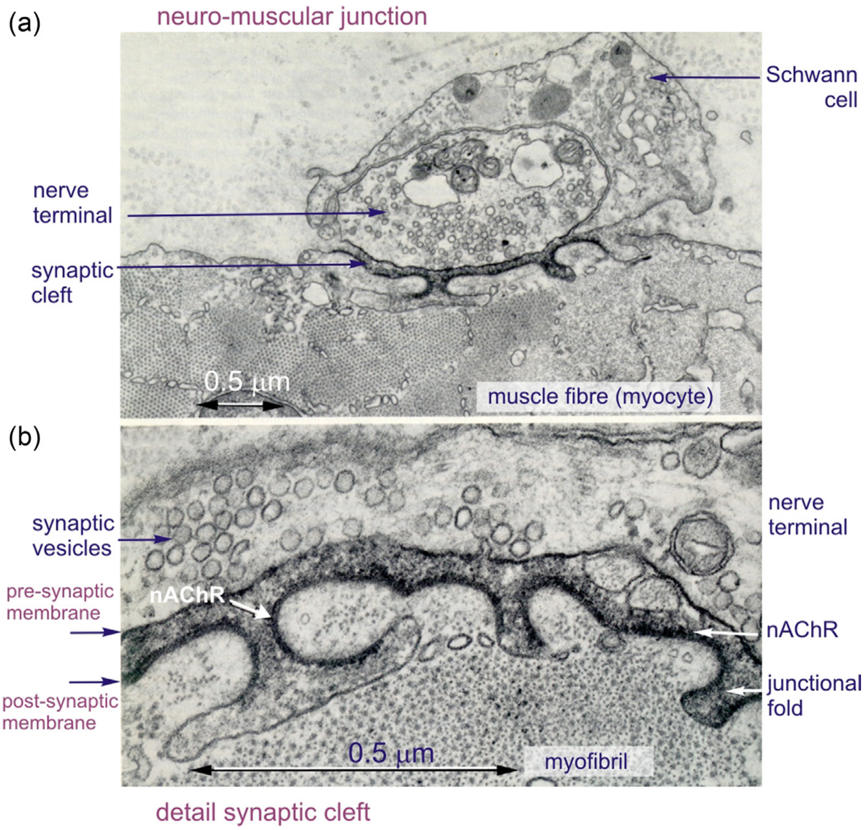


FIGURE 4-4 Thin section electron micrographs demonstrating the presence of nicotinic acetylcholine receptors in the frog cutaneous pectoris muscle. (a) The nerve terminal, with mitochondria and numerous synaptic vesicles, is encased by a Schwann cell and abuts a muscle fiber. The preparation was treated with α-bungarotoxin fused with horseradish peroxidase (highly selective for muscle-type nicotinic receptors), followed by staining with 3,3′-diaminobenzidine to reveal the receptors as an electrodense reaction product. (b) The zoom shows that the staining of nicotinic receptors can be seen to be mainly confined at the extracellular surface of the postsynaptic membrane with some diffusion into the synaptic cleft. *Image from* Burden et al. (1979).

develops from cylindrical myotubules (classic striated muscle tissue) which transit into cup-shaped cells called the "electrocytes." These electrocytes organize in stacks and these constitute the electric organ. Somatic motor neurons that liberate acetylcholine do not induce contraction but instead cause an "electric shock" due to massive opening of the ion channels that give rise to a positive (Na^+) current (Fox and Richardson, 2004). Although electron microscopy provides a low-to-medium resolution approach, applying crystallographic refinement methods to the data has yielded a structure having atomic resolution (Unwin, 2005).

Architecture of the neuromuscular junction nicotinic receptor

Five subunits are arranged around a central pore. When viewed from the synaptic cleft the clockwise sequence is α1,γ,α1,β1,δ. The overall shape of the receptor is a hollow cone, approximately 8 nm in diameter at its widest point and some 16-nm long. The assemblage protrudes into the synaptic cleft and within this protuberance there is a central vestibule. The N-terminal extracellular domain contains the ligand-binding sites and the highly conserved disulfide pair that forms a so-called cys-loop, which, as we mentioned before, is a characteristic of a number of ligand-gated ion channels (Connolly and Wafford, 2004). On the intracellular side of the membrane, the structure protrudes into the cytoplasm and contains another smaller cavity with side openings. The two cavities communicate through the central pore (Figure 4-5).

Each subunit traverses the membrane four times (M1–M4) by means of classic hydrophobic α-helices. The extracellular segment is characterized by extensive β-strands, packed into a curled sheet, and one α-helix. The characteristic cysteine bond plays an important role in stabilizing the packed curled β-sheet and, as we will see later, this provides a certain rigidity that helps to transmit changes that occur at the level of ligand binding, into changes in ion-conductivity. A number of flexible connecting loops between the β-strands contribute to ligand binding. These loops are named A, B, and C for the principal α-subunit (in this example α1) and loops D and E for the complementary subunit (in this example δ or ε) (Figures 4-6 and 4-7). Acetylcholine, or its agonist nicotine, is normally sandwiched between the residues tyr-93 (principal α1-subunit) and trp-54 and leu-118 (complementary subunits). The binding site is best described as an "aromatic cage" (Figure 4-7; Brejc et al., 2001). Two ligand-binding sites must be occupied in order to open (activate) the receptor. The three-dimensional structure shown does not contain acetylcholine, it represents the receptor in its closed (inactive) state.

The loops connecting the transmembrane spans are relatively short (loop M1-M2 on the inside of the cell and M2-M3 on the outside). Importantly, the M2-M3 loop tightly interacts with the cys-loop underneath the curled β-sheet. The negatively charged glu-45 (cys-loop) interacts with the positively charged pro-272 (on M2-M3 loop) and the nonpolar val-46 (cys-loop) docks into a hydrophobic pocket provided by ala-270 and val-271 (on M2). Together (M2-M3 and cys-loop) they constitute a rather rigid linker that could transmit conformational changes of the ligand-binding domain to the pore lining M2-helix (Figure 4-6(c)). The sequence connecting M3 with M4 is more extended and it makes up the bulk of the intracellular segment. The sequence is partly folded into the MA helix and partly disordered (dotted line Figure 4-6(c)) and therefore not traceable in the electron-density map.

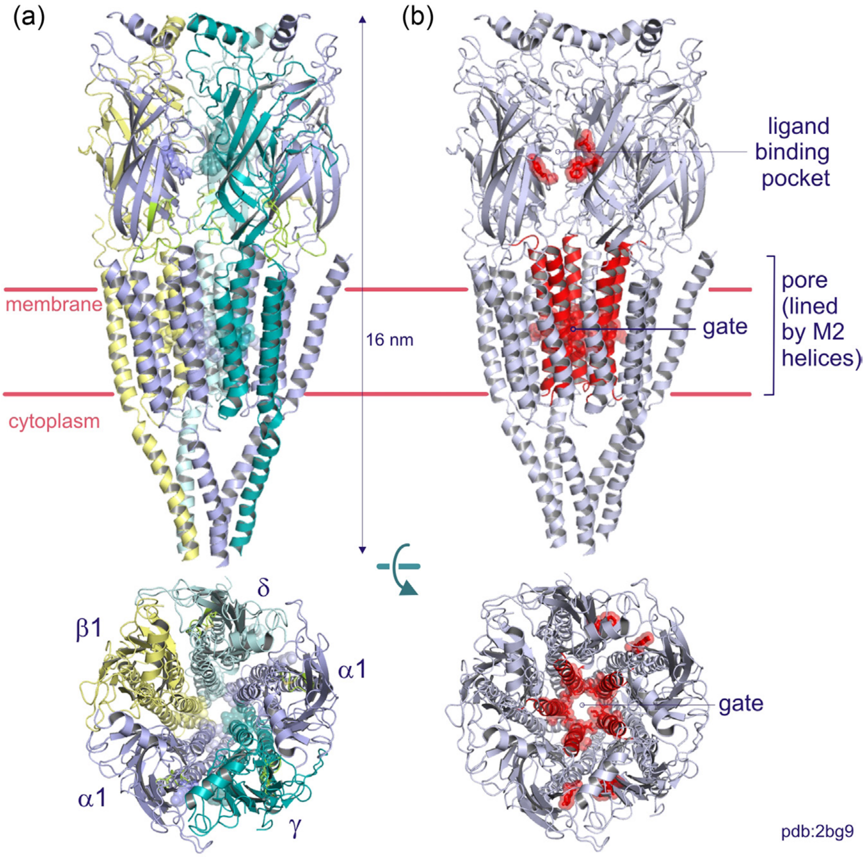
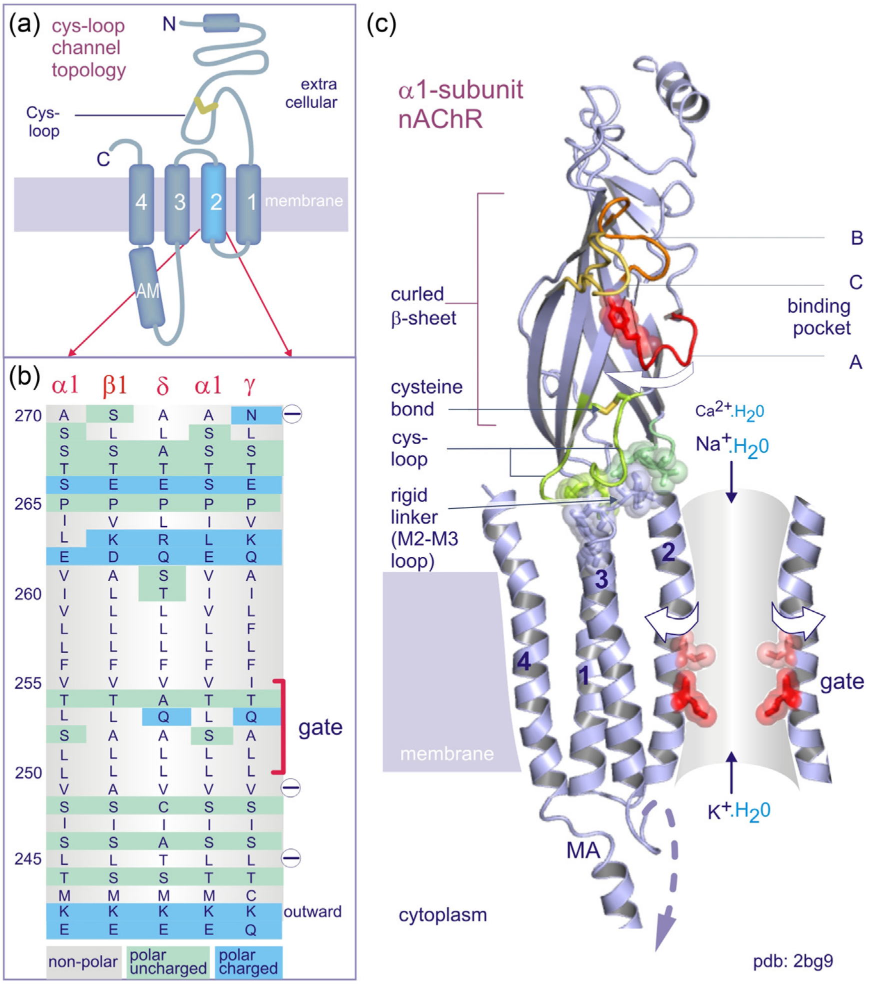


FIGURE 4-5 Global structure of the nicotinic receptor without ligand. (a) Side view and view from the synaptic cleft illustrating the pentameric composition surrounding a central pore. (b) This representation of the receptor highlights one of the two ligand-binding pockets, in the extracellular segment, and the transmembrane helices that line the channel pore (M2, in red). The narrowest point, halfway the membrane passage, constitutes the channel gate. In the absence of acetylcholine, it is not permeable to ions which are accompanied by their hydration shell. Pdb: 2bg9.

M2 sequences line the pore

The M2 helices that shape the conduction path of the channel are slightly kinked. These helices traverse the membrane in register with sets of homologous residues at each level forming rings of chemically distinct environments facing the lumen of the pore (Figure 4-6(b)). These rings are predominantly nonpolar, and present a relatively inert surface to diffusing ions, but three of them contain negatively charged groups (from outside to inside, rings containing E241, E/D262, and S/E266). These are likely to constitute the ion-selectivity filter, that is,



	α1	β1	δ	α1	γ	
270	A	S	A	A	N	⊖
	S	L	S	S	L	
	S	S	A	S	S	
	T	T	T	T	T	
	S	E	E	S	E	
265	P	P	P	P	P	
	I	V	L	I	V	
	L	K	R	L	K	
	E	D	Q	E	Q	
	V	A	S	V	A	
260	I	L	T	I	I	
	V	L	L	V	L	
	L	L	L	L	F	
	L	L	L	L	L	
	F	F	F	F	F	
255	V	V	V	V	I	gate
	T	T	A	T	T	
	L	L	Q	L	Q	
	S	A	A	S	A	
	L	L	L	L	L	
250	L	L	L	L	L	
	V	A	V	V	V	⊖
	S	S	C	S	S	
	I	I	I	I	I	
	S	S	A	S	S	
245	L	L	T	L	L	⊖
	T	S	S	T	T	
	M	M	M	M	C	
	K	K	K	K	K	outward
	E	E	E	E	Q	

non-polar · polar uncharged · polar charged

FIGURE 4-6 Structure–function analysis of the ligand-binding and ion-conducting properties of the nicotinic acetylcholine receptor. (a) Characteristic topology of subunits of cysteine-loop ligand-gated ion channels. Five such subunits constitute the ion channel (pentamer). (b) Sequence alignment of M2 helices of the *Torpedo* nAChR illustrates the sets of homologous residues that form rings of chemically distinct environments facing the lumen of the pore. Three rings of negatively charged residues are alternated by polar uncharged and nonpolar residues. The negative charges are thought to play a role in cation selectivity of the channel. The narrowest part of the pore, stretching from L250 to V255, constitutes the gate (0.8-nm diameter). Ions accompanied by their hydration shell are too large to cross the pore. The pore environment is entirely unsuitable for ions to cross the gate alone because of a total lack of electrostatic interactions that could replace water. (c) Detail of the α1-subunit of the nAChR of *Torpedo*. The extracellular segment contains the primary ligand-binding site, to which three loops participate: A, B, and C. An essential ligand-binding tyrosine, in loop A, is shown in red. The cysteine-loop (cys-loop) contacts transmembrane helices M2 and M3, forming a rather rigid linker between the extracellular and transmembrane segment. Conformational changes induced by binding of acetylcholine, constituting a rotation of the curled β-sheet, lead to a positional change of the M2 helices (transmitted through a rigid linker composed of the M2-M3 loop). This increases the aperture of the pore as well as the identity of the amino acids that face the lining of the channel, resulting in a flow of cations (Na$^+$, Ca^{2+}, and K$^+$) accompanied by water. Pdb: 2bg9.

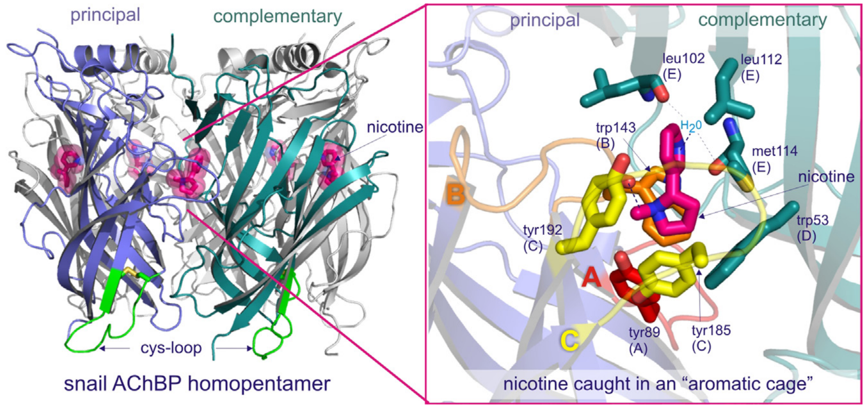


FIGURE 4-7 The snail acetylcholine-binding protein (AChBP), which assembles into a homopentamer, serves as a template to better understand the operating mode of cys-loop ligand-gated ion channels. With respect to pharmacological and sequence similarity, the complex resembles a human α7-homopentamer providing five ligand-binding pockets but the protein lacks the pore-forming section (compare with Figure 4-5). The five nicotines are coordinated by "principal" and "complementary" contacts at the interface of two subunits. The zoom shows the "aromatic cage" that surrounds nicotine. The contribution of the principal subunit comprises tyr-89 (A-loop), trp-143 (B-loop), tyr-192, and tyr-185 (C-loop). The complementary subunit contributes with leu-102, leu-112, met-144 (all E-loop), and trp-53 (D-loop). The majority of interactions are hydrophobic (leucine, methionine) or pi-stacking (tyrosine, tryptophan), with the addition of two hydrogen bonds, that employ a water intermediate (leu-102 (E-loop), met-114 (E-loop)). Pdb: 1uw6.

enriching positively charged ions (cations), and reducing negatively charged ions (anions). All subunits contain a highly conserved positively charged lysine (K242), at the bottom of M2, but this faces outward, away from the pore and into the direction of M3. The sparse charged residues situated in a globally nonpolar environment sets the condition for rapid transport because traversing ions and water have really little to hold on to (and are thus not slowed down). A similar strategy is developed for aquaporin, a fast conduction water channel (Murata et al., 2000). The gate is formed by a narrow ring, with an inner diameter of roughly 0.8 nm, constituted by residues between leu-251 and val-255. This pore is sufficiently wide to let Na^+, K^+, or Ca^{2+} through (diameters in the range 0.3–0.5 nm) but too constricting when these ions are accompanied by a first hydration shell (>0.8-nm diameter). As ions will refuse to travel through the pore in the absence of ionic contacts (that mimic the hydration shell), flow arrests here. Hence, this region can be identified unambiguously as the gate of the pore (Figure 4-6; Heidman and Changeux, 1984; Miyazawa et al., 2003; Unwin, 2005; Celie et al., 2004).

Gating mechanism

Electron microscopic analysis has indicated that the pore widens in the middle of the membrane when the receptor is activated, implying that the hydrophobic girdle, forming the gate, comes apart to allow water-surrounded cations to flow through (Unwin, 1995; Unwin et al., 2002). The structural details of the closed receptor imply a simple mechanical model for channel opening. First, the conformational change in the ligand-binding domain, which affects primarily the α-subunits, brings about the rotations of their inner β-sheet. Second, the rotations of the β-sheet are transmitted to the M2 helices surrounding the gate. Third, the ensuing twisting movement of M2 weakens the hydrophobic side-to-side interactions that hold the gate together. As a result, the helices "collapse" back against the outer protein wall, making alternative hydrophobic contacts in a configuration that is permeable to the cations; as a consequence of widening the lumen of the gate and changing the contact surface (Figure 4-6(c)). Molecular detail is still lacking to substantiate the precise mechanism of rotation of the M2 helix.

Snails help to reveal molecular details of the acetylcholine receptor

The structure we have presented is obtained from a large range of experimental data, biochemical and structural, which are mapped on a three-dimensional framework provided by hundreds of electron microscopy images of the frozen receptors from *Torpedo* membranes in a near-physiological ionic environment. Much detailed molecular information of the ligand-binding segment has been obtained thanks to a soluble truncated acetylcholine receptor occurring in glia cells of the great pond snail, *Lymnaea stagnalis*. Like genuine nicotinic receptors, this acetylcholine-binding protein (AChBP) assembles into a homopentamer with ligand-binding characteristics that are typical for a nicotinic receptor but, unlike the nicotinic receptor, it lacks the domains to form a transmembrane ion channel (Smith et al., 2001). It is secreted into the synaptic cleft by glia cells upon synaptic excitation of neurons by acetylcholine. Its presence serves to modulate cholinergic synaptic efficacy, possibly by sequestration of acetylcholine (Sixma and Smith, 2003). The protein is most closely related to the human α7 subunit, both with respect to pharmacological profile and amino acid sequence. The 0.27-nm resolution structure of AChBP is extensively used as a structural template to better understand the functioning of cys-loop ligand-gated ion channels (acetylcholine, GABA, 5HT3, and glycine receptors) (Celie et al., 2005). The snail AChBP is currently an important tool in the analysis of novel modulators of the nicotinic receptor (Figure 4-7).

- Web resource of ligand-gated ion channels http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php
- Web resourse Wikipedia "nicotinic acetylcholine receptor"

Receptor desensitization

When a nicotinic receptor is exposed to a high concentration of acetylcholine, it quickly activates and the channel opens (within a millisecond) and then closes. In the continued presence of ACh, the channel can repeatedly open and close but over a period of a few hundreds of milliseconds to seconds the receptor undergoes a slower conformational change through which it enters a refractory or inactive closed channel state. This phenomenon is referred to as desensitization. At the neuromuscular junction, such desensitization will result in muscle paralysis and so prevent breathing. This is why cholinesterase inhibitors are so dangerous because inhibition of cholinesterase at the neuromuscular junction will result in prolonged high concentrations of acetylcholine in the synaptic cleft (Katz and Thesleff, 1957; Rang and Ritter, 1970; Magleby and Pallotta, 1981; Unwin et al., 1988). Suxamethonium and decamethonium are agonists that cause muscle relaxation through the above described desensitization process. They are used in surgery.

α-Bungarotoxin is a 74 amino acid peptide obtained from the venom of the snake *Bungarus multicinctus* (Taiwanese banded krait). It binds specifically and with high affinity to the neuromuscular-and to the monomeric-type (α7, α9, or α10) nicotinic receptors ($K_D = 1\,\text{nmol/l}$), with consequent inactivation (a paralysis of the prey). In the laboratory, the toxin can be immobilized by attachment to a solid phase (such as Sepharose beads). By applying affinity chromatography, using column packed with these beads, it was possible to purify nicotinic receptors, after solubilization in detergent, from muscle or electric-organ membranes, well before the advent of DNA cloning (Changeux et al., 1970). The toxin is also used in staining methods to detect the presence of the nicotinic receptor (see Figure 4-4).

Patch clamp electrophysiology to study behavior of ligand-gated ion channels

With the introduction of the patch clamp technique by Neher and Sakman (Neher and Sakman, 1976), it became possible to study individual ion channels in situ in a membrane. It is hard to overestimate the contribution that this advance has made to our understanding of channel mechanisms. The idea itself is simple enough. A patch pipette, which is a glass microelectrode with a smooth (fire-polished) tip, a few micrometers in diameter, is pressed gently against the outside of a cell. With gentle suction, a very tight seal forms between the pipette tip and the plasma membrane. The seal resistance is typically more than a gigohm ($10^9\,\Omega$). Two alternative maneuvers can then be used to remove a small area of membrane from the cell without breaking the seal (Figure 4-8). The end result is either an outside-out or an inside-out patch of membrane. These contain only a very few ion channels, but depending on the orientation, the ligand-binding sites are either exposed to the exterior or to the contents of the patch pipette. The paucity of channels within the patch and the high-resistance seal then enable recordings of membrane current to be made, for example, under voltage-clamp conditions. These reveal individual channel openings and closures.

Nicotinic receptors were among the first channels to be characterized in this way. Like other ion channels, they open and close abruptly in an all-or-none fashion and they do this spontaneously. The effect of acetylcholine is to increase the probability that a particular channel is in its open state.

The study of synaptic transmission and the role of the nicotinic acetylcholine receptors has been rewarded by three Nobel Prizes in Physiology or Medicine. In 1936, the Prize was awarded jointly to Henry Hallett Dale and Otto Loewi "*for their discoveries relating to chemical transmission of nerve impulses*," in 1970 to Bernard Katz, Ulf von Euler, and Julius Axelrod "for their discoveries concerning the humoral transmitters in the nerve terminals and the mechanism for their storage, release, and inactivation" and in 1991 to Erwin Neher and Bert Sakman "*for their discoveries concerning the function of single ion channels in cells*."

MUSCARINIC ACETYLCHOLINE RECEPTORS

Whereas acetylcholine acts through the nicotinic receptor to stimulate contraction of skeletal muscle, it decreases the rate and force of contraction of heart muscle through the muscarinic receptor (type M2). The muscarinic acetylcholine receptors belong to the seven transmembrane-spanning (7TM) superfamily of G-protein-coupled receptors and they exist as five subtypes, M1–5 (Figure 4-2; Haga et al., 1985; Haga and Haga, 1985; Kubo et al., 1986). Muscarinic receptors signal through G-proteins and bring about a physiological response through the production of second messengers. They are

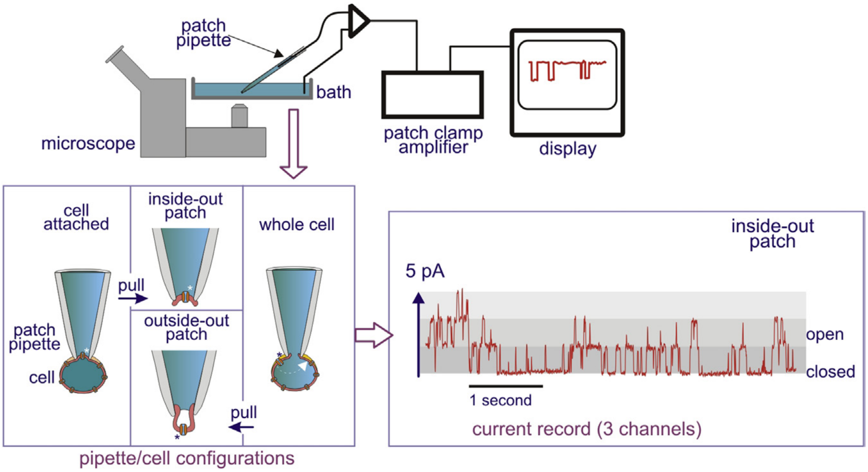


FIGURE 4-8 Using membrane patches to record single channel opening and closure. Typically, cells are allowed to adhere to the bottom of a shallow dish. Individual cells are then attached to a patch pipette. A typical current record obtained from a patch of membrane, detached from a cell, is shown.

therefore also referred to as metabotropic receptors (activating metabolic pathways that generate second messengers). Muscarinic receptors have many actions, dependent on the tissues to which they are applied and the receptor subtypes that are expressed. With respect to molecular structure, the different subtypes show a very high degree of sequence similarity in the transmembrane core and this may explain why there are only few receptor-specific agonists, reverse agonists, or antagonists (Conn et al., 2009).

The molecular structure of the M2 and M3 receptors has been resolved and together with the application of dynamic modeling they have revealed two novel aspects (Haga et al., 2012; Kruse et al., 2012). First, the M2 receptor exhibits many features noted previously as common structural elements in unrelated acetylcholine-binding proteins. Like the snail nicotinic acetylcholine-binding protein, the M2 receptor shows an aromatic cage comprising three tyrosines and a tryptophan. Such features are also found in the acetylcholinesterase of *Torpedo californica* and the bacterial ChoX acetylcholine-binding protein (Figure 4-9). Second, molecular dynamics simulations of the path by which the inverse agonist tiotropium binds to and dissociates from M2 and M3 receptors revealed two topographically distinct binding sites: one at the extracellular surface and one inside the transmembrane region. Mutation analysis has shown that receptor activity is dependent on certain residues that make up the external binding site (Redka et al., 2008; Kruse et al., 2012). Tiotropium is said to act as a bitopic ligand, binding and acting at two different sites. The external binding site has much lower subtype-sequence conservation and conceivably this site could be exploited for the design of therapeutic molecules that are much more subtype specific (Figure 4-10; Conn et al., 2009).

ALLOSTERIC AND ORTHOSTERIC REGULATION

If we consider receptors as enzymes, and replace ligand with substrate-binding site, than we can propose two types of regulation. One is determined by binding of the substrate to the enzyme, the other by allosteric regulation through metabolic intermediates or end products which control enzyme activity at sites remote from the substrate-binding site. Tiotropium is thought to do both. It regulates activity when situated in the transmembrane region and when fixed to its external binding site. To make the distinction, two new terms have been introduced. We speak of orthosteric binding sites and orthosteric regulation when it concerns occupation of the classic ligand-binding site, and we speak of allosteric binding sites and allosteric regulation when it concerns external binding sites. For receptors with high sequence homology, allosteric binding sites are attracting much interest because they may be less conserved among receptor subtypes (Dror et al., 2013). Importantly, allosteric regulation may also occur through interaction with other receptors, for instance, those that form dimers or trimers, or with other, unrelated, membrane or cytosolic proteins.

Muscarinic receptors can be divided into two subgroups: the uneven numbers (M1,3,5) interact with members of Gαq/11 and signal predominantly through activation of phospholipase C, and the even numbers (M2,4) interact with members of Gαi/o and signal predominantly through inhibition of adenylyl cyclase (Figure 4-11). In the case of the M2 receptor in the heart, the Gβγ subunit, functionally liberated upon Gα-activation, directly binds potassium channels and augment their conductance, leading to an increased outward K^+ current (Wickman et al., 1994). It should be noted, however, that the selectivity for G-protein is not strict. For instance, interactions of M1, -3, or -5 with other G-proteins (Gi/Go/Gs) can be readily observed in cells expressing recombinant receptors, even at relatively low levels (Nahorski et al., 1997). There is not enough structural information to answer the question of how different muscarinic receptors determine their choice of G-proteins. A first hint is given by the observation that the distance

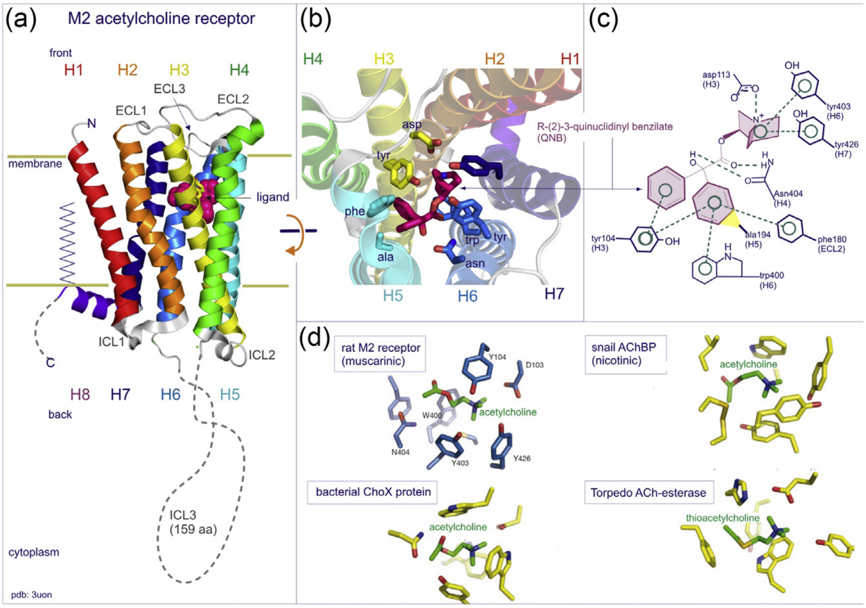


FIGURE 4-9 Structure of the muscarinic acetylcholine receptor (type M2) bound to the antagonist QNB. (a) Typical 7TM architecture of the M2 receptor. The relative position of the transmembrane helices resembles that of adrenoceptors (return to Chapter 3, Figure 3-4) with three major exceptions, the muscarinic receptor has only a short 2nd extracellular loop (ECL2) and a very short C-terminal segment (following H8) but, instead, they possess a very long 3rd intracellular loop (ICL3, counting 159 amino acids). (b) The ligand, the antagonist QNB, is buried in the transmembrane section and coordinated by helices 3, 5, 6, and 7. (c) Detail of the interactions between ligand and receptor. Note that the majority are of the type pi–sigma stacking with the multiple tyrosines and one tryptophan constituting a so-called "aromatic cage." Asp-113 makes ionic interactions (a salt bridge) and asn-404 provides two hydrogen bonds. (d) Comparison of the acetylcholine-binding pocket of four unrelated proteins (a receptor (rat), an enzyme (*Torpedo*), and two binding proteins) reveals a conserved composition of the "aromatic cage." Interactions are primarily a mixture of pi and sigma stacking. It should be noted that the snail AChBP serves as a model for the nicotinic receptor. *Image adapted from Haga et al. (2012) with permission.*

between TM5 and TM6, at the cytosolic side, is larger (by ~0.4 nm) for the Gαi/o-coupled receptor (M2) than for the Gαq/11-coupled receptors (M3) (Kruse et al., 2012).

In the next sections, we place the acetylcholine receptors in the context of skeletal muscle (type IV nicotinic), cardiac muscle and pacemaker (M2 muscarinic), airway smooth muscle (M3), and vascular smooth muscle (M3). The control of vascular muscle tone also occurs through acetylcholine-mediated release of nitric oxide (NO) from endothelial cells and we will elaborate on the role of NO in smooth muscle cell relaxation.

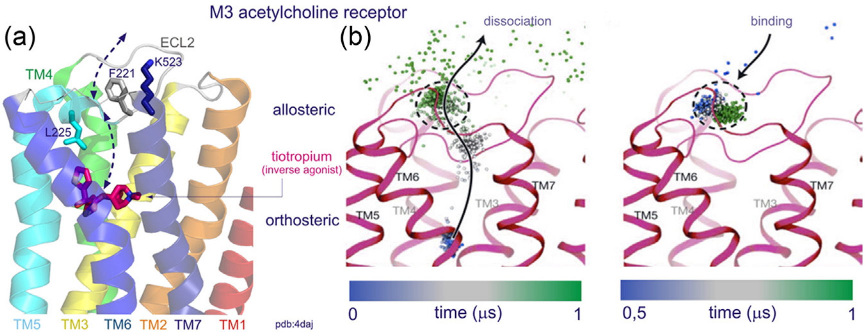


FIGURE 4-10 Bitopic ligand-binding of the inverse agonist tiotropium to the M3-acetylcholine receptor. (a) Structure of the M3 receptor with tiotropium in the binding pocket (orthosteric site). A second binding site (allosteric or external site) occurs at the entry of the vestibule of the receptor. Key residues that control binding at the external site are highlighted in sticks (F221, L225, and K523). (b) Dynamic simulation of tiotropium dissociation from or binding to the M3 receptor. When starting the simulation with tiotropium bound to the pocket, rapid dissociation is observed with a transient slow-down at the allosteric binding site (going from blue, to gray to green in 1 μs). When the ligand is placed outside the receptor, it only reaches the external binding site in the same time span. The simulation is still too short in order to reveal binding to the orthosteric site. Conformational changes of the receptor occur at both poses. *Image adapted from Kruse et al. (2012).*

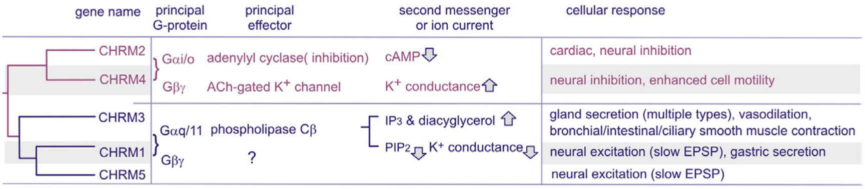

gene name
principal G-protein
principal effector
second messenger or ion current
cellular response
CHRM2
CHRM4
Gαi/o
Gβγ
adenylyl cyclase(inhibition)
ACh-gated K+ channel
cAMP ⇩
K+ conductance ⇧
cardiac, neural inhibition
neural inhibition, enhanced cell motility
CHRM3
CHRM1
CHRM5
Gαq/11
Gβγ
phospholipase Cβ
?
IP3 & diacylglycerol ⇧
PIP2 ⇩ K+ conductance ⇩
gland secretion (multiple types), vasodilation, bronchial/intestinal/ciliary smooth muscle contraction
neural excitation (slow EPSP), gastric secretion
neural excitation (slow EPSP)

FIGURE 4-11 Signaling modes of muscarinic receptors. The M2 and M4 predominantly signal through Gαi or Gαo and they inhibit adenylyl cyclase, hence reduce the intracellular cAMP content. Their Gβγ subunits enhance the outward conductance of K^+ channels and this provides a generally inhibitory effect on excitatory cells. For some epithelial cells, the inhibition of cAMP causes an increased motility. The M1, M3, and M5 signal predominantly through Gαq and Gα11 and they stimulate phospholipase C, leading to the production of IP_3 and diacylglycerol (DAG). In neurons, the concomitant reduction in PIP_2 (substrate of the enzyme) reduces outward K^+ conductance and this gives rise to a slow excitatory postsynaptic potential (EPSP). Numerous concomitant EPSPs may lead to an action potential (signal transmission in nerves). We have not found a role for Gβγ associated with Gαq/11 signaling.

TYPE IV NICOTINIC AChR INDUCES SKELETAL MUSCLE CONTRACTION

Somatic efferent motor neurons terminate on skeletal muscle in specialized neuromuscular junctions or end plates. Upon the arrival of an action potential, acetylcholine is released and traverses the synaptic cleft. The myocytes express the type IV nicotinic AChR and these rapidly open. The channel is permeable to Na^+ and K^+ but in first instance only Na^+ enters the cell and causes a partial depolarization of the membrane potential (upstroke or end plate potential). As a consequence, voltage-sensitive Na^+ channels (SCN4A or NAv1.4) open and the membrane potential reverses to +30 mV (action potential). Membrane depolarization inactivates the Na^+ conductance through a plug tethered to the channel. The excitation–contraction coupling follows a short pathway starting with a membrane-voltage-induced conformational change in a voltage-gated Ca^{2+} channel (CACNA1C or Cav1.2 sensitive to dihydropyridine) which, in turn, provokes the opening of the ryanodine-sensitive Ca^{2+} channel in the sarcoplasmic reticulum (RYR1). Inflowing Ca^{2+} binds the troponin-C subunit (TNNC2) and this leads to a displacement of the troponin-I subunit (TTNI2), thus allowing myosin-II to bind to actin (return to Chapter 3, Figure 3-19). Typically, intracellular free Ca^{2+} concentration may rise 100-fold, from 50 nM at rest to 5 μM during contraction. The cross-bridge cycle sets off and lasts until Ca^{2+} is removed from the cytosol by a Ca^{2+} pump (ATP2A1 or SERCA1). Unlike the heart, skeletal muscle does not express phospholamban and Ca^{2+} uptake is solely controlled by the Ca^{2+} concentration itself. Alternative transporters for Ca^{2+} are the plasmalemma-localized Ca^{2+}-ATPase (ATP2B2), weakly expressed in muscle, and the Na^+/Ca^{2+} exchanger (SLC8A1 or NCX1). Membrane repolarization occurs through the sortie of K^+ through two types of voltage-gated channels, KCNA4 (Kv1.4 or Shaker-type) and KCNC4 (Kv3.4 or Shaw-type) which are mainly expressed in the tubular system (DiFranco et al., 2012). The Ca^{2+} stores are refilled by the Ca^{2+}-ATPase (ATP2A1) which takes source from Ca^{2+} entering the cell through store-operated Ca^{2+} channels (ORAI1) which are associated with, and controlled by, the Ca^{2+} sensor STIM1 (Figure 4-12; Stiber and Rosenberg, 2011). The refractory period of the voltage-sensitive Na^+ is short (lasting a few milliseconds) and repeated stimulation, from 10 Hz onward, causes summation of contraction (the muscle fiber no longer fully relaxes).

Regulation of contractile force in skeletal muscle differs from heart muscle because of a shorter refractory period and a different mode of innervation. Both muscle types utilize the intracellular free Ca^{2+} to regulate actin–myosin cross-bridge intensity and thus strength of contraction. However, whereas the heart necessarily relaxes between two beats, skeletal muscle can be stimulated repeatedly during a contraction wave, causing the Ca^{2+} to accumulate. This leads to an increased and steady tension (tetanus), a phenomenon described as “frequency summation.” Moreover, skeletal muscle has the possibility to mobilize different numbers of fibers because contraction is organized in motor units. These are

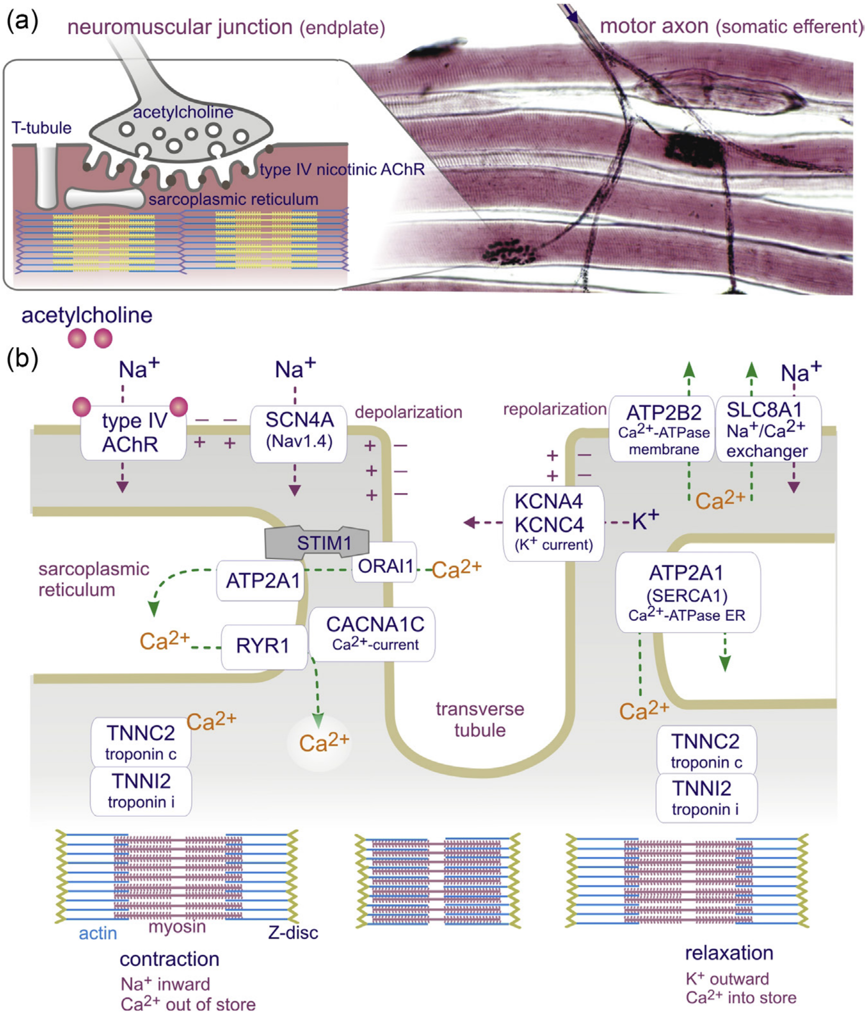


FIGURE 4-12 Skeletal muscle excitation–contraction scheme. (a) Motor neurons terminate on the skeletal muscle fiber (which is a fusion product of thousands of myocytes) in a specialized synapse, called the neuromuscular junction or end plate. (b) Acetylcholine binding to the type IV nicotinic ACh receptor opens the channel (permeable for Na^+, K^+, and to a much lesser extent Ca^{2+}) and in first instance this gives rise to an inward Na^+ current. The ensuing partial depolarization opens voltage-sensitive Na^+ channels (SCN4A) which induce an action potential. Voltage-gated Ca^{2+} channels (CACNA1C) respond with a change in conformation which is translated into the opening of the ryanodine receptor (RYR1). Ca^{2+} now enters the cytoplasm from the sarcoplasmic reticulum and it binds troponin-c, leading to the displacement of troponin-I, thereby, allowing myosin-II to interact with actin and starting the cross-bridge cycle. The process is arrested by the repolarization of the membrane potential through the outflow of K^+ via KCNA4 and KCNC4 channels. This prompts the entry of Ca^{2+} and allows the Ca^{2+}-ATPase of the sarcoplasmic reticulum (ATP2A1) to remove the Ca^{2+} from the cytosol. Alternative transport mechanisms are a membrane bound Ca^{2+}-ATPase (ATP2B2) and an Na^+/Ca^{2+}-cotransporter (SLC8A1). Intracellular Ca^{2+}-stores are replenished through the entry of extracellular Ca^{2+} via a storage-operated Ca^{2+}-channel (ORAI1). Its conductance is under control of the Ca^{2+}-sensor STIM1.

defined as a set of fibers innervated by a single (but branched) motor neuron (unitary mode of innervation). Thus, a weak signal will mobilize small motor units but if you have to lift up a larger load, more and larger motor units are activated. This process is referred to as "fiber summation." The number of motor units that innervate a muscle is difficult to estimate but ranges from a few tens, example of the extensor pollicis longus muscle which controls movement of the thumb, to several hundred, example of biceps brachii muscle (widely known as the biceps) (Heckman and Enoka, 2012). Generally, we do not mobilize more than 30% of our muscle at any time, motor units alternate their force generation (Unglaub-Silverthorn, 2010).

Tetanus toxin is produced by *Clostridium tetani*, an obligate anaerobic bacterium that enters the body through deep wounds (rusty nails seem a favorable place for the spores of the bacterium). After an incubation period of one or several weeks, the toxin enters motor neurons and travels retrograde to the spinal cord synapses. It then switches to presynaptic ganglions where it causes proteolysis of SNAREs, proteins involved in neurotransmitter secretion. Two neurotransmitters are particularly concerned: glycine and γ-aminobutyric acid, both of which inhibit motor nerve conduction. Loss of their inhibitory control causes severe contractions (muscle spasms) first manifested by involuntary facial- and jaw spasms (lockjaw). Due to the lack of inhibitory control, muscle spasms caused by tetanus toxin may exceed tendon and bone strength and cause rupture and fracture. The name tetanus comes from ancient Greek, meaning taut or tense.

The events underlying excitation–contraction coupling in muscle were for the first brought to light in giant striated muscle fibers of the acorn barnacle *Balanus nubilus* (Ashley and Ridgway, 1968). To achieve this, Ashley and Ridgway had injected a Ca^{2+}-sensitive bioluminescent protein, aequorin (purified from the medusa *Aequorea aequorea*) into the fiber and by measuring simultaneously contraction, fluorescence, and membrane potential, they revealed the characteristic relationship of 1st an action potential, followed by an increase in intracellular free Ca^{2+} and then muscle contraction. Muscle tension reached its maximum when the cytosolic free Ca^{2+} concentration was already returned to baseline.

ACETYLCHOLINE, ACTING ON THE M2-RECEPTOR, REDUCES FORCE AND SLOWS DOWN THE HEART RATE

We already mentioned that the heart is innervated by the parasympathetic system via the vagus nerve, which inhibits the rate. The vagus nerve terminates on various ganglia, both on the atria, near the AV node, and the ventriculi. From these ganglia, postsynaptic nerves reach more or less the whole of the heart and, importantly, the SA node (Coote, 2013). The nerve endings release acetylcholine (return to Chapter 3, Figure 3-2). The heart expresses the muscarinic M2 receptor which connects with members of the Gαi/o family of which Gαi1, -2, and -3 (GNAI1-3) are present in the heart (return to Chapter 2, Figure 2-29). Activation of Gi has two consequences. First, Gαi1 binds and inhibits adenylyl cyclase (of which ADCY5 is most active in the heart). As a result, acetylcholine reduces both basal- and noradrenaline-mediated production of the second messenger cAMP and thus weakens contraction force and reduces automaticity (Figure 4-13). Indeed, inactivation of Gαi by pertussis toxin causes an increase in myocyte contractility and automaticity (Xiao et al., 1998). As a consequence of inhibition of ADCY5, depolarization-mediated Ca^{2+} entry is reduced, resulting in a lower level of intracellular free Ca^{2+}. For a description of the action of adrenaline return to Chapter 3, Figure 3-22. A second important consequence concerns the pacemaker cells. The functionally liberated Gβγ subunits bind to inward-rectifier K^+-channels (KCNJ3 or GIRK1) and increase their conductivity. Evidence has been provided that binding of multiple Gβγ-subunits brings about a rotation movement between the cytosolic segments of the channel. This movement opens the channel (Figure 4-14; Logothetis et al., 1987; Luscher and Slesinger, 2010; Whorton and MacKinnon, 2013). Under physiological conditions, this translates into an increased outward K^+ current which lengthens the pacemaker-potential phase because it opposes the slowly depolarizing inward Na^+ current (funny current) mediated by HNC4 (Figure 4-13(a)). As a result, the pacemaker cells fire less frequently, thus slowing down the heart rate (Figure 4-13(d); Postea and Biel, 2011). Lastly, the increased K^+ outward and decreased Ca^{2+}-inward conductance both contribute to a conduction block at the AV node, where propagation of the action potential depends on Ca^{2+} current (not Na^+).

In the Chapter 3, we hinted to the detrimental effect of a high sympathetic tone (elevated noradrenaline level) on the heart (causing cell death) (Brook and Julius, 2000). An elevated resting heart rate (low parasympathetic tone) is a risk factor too. It was shown among a population of 2800 Danish citizen, who were followed for a period of 16 years, that those with an elevated resting heart rate (>90 beats per minute) had a significantly higher mortality than those with a low rate (<50 beats per minute). The mortality rate is independent of physical fitness, leisure-time

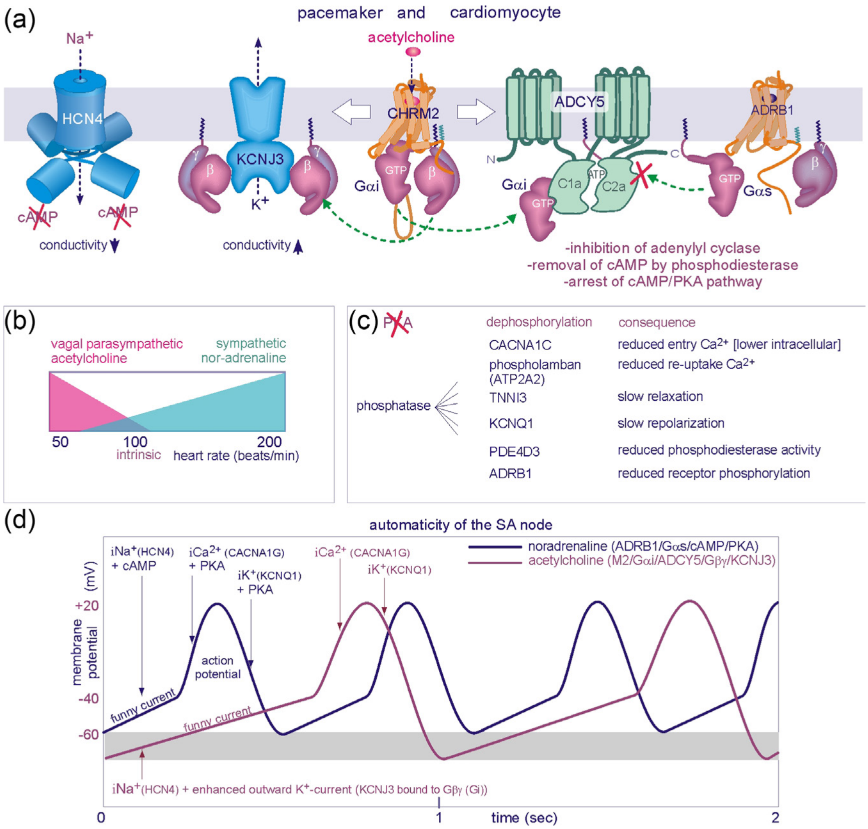


FIGURE 4-13 Vagal (parasympathetic) control of force and rhythmicity of the heart through acetylcholine signaling via the muscarinic-M2 receptor. (a) Gαi-mediated inhibition of adenylyl cyclase reduces both basal- and noradrenaline-stimulated production of cAMP and this reduces muscle force. Acetylcholine has an important effect on rhythmicity of the pacemaker cells first because the loss of cAMP causes a reduction of conductivity of the inward Na^+ funny current (mediated by HCN4) and second because functionally separated Gβγ subunits interact with the inward-rectifier K^+ channel (KCNJ3) and cause an increased outward current. The increased K^+ outward current opposes the slow depolarization effect of the funny current (see below) and thus reduces the slope of the pacemaker potential. (b) Vagal parasympathetic and sympathetic control of the heart rate. Without innervation the heart rate is around 100 beats/min. Acetylcholine reduces, whereas adrenaline increases rhythmicity. (c) The acetylcholine-mediated reduction of cAMP and subsequent loss of PRKAC (PKA) activity leads to phosphatase-mediated dephosphorylation of CACNA1C and phospholamban, thereby, limiting the influx of Ca^{2+} and its reuptake into the sarcoplasmic reticulum respectively. Dephosphorylation of troponin-c causes an increase in the affinity for Ca^{2+}, giving rise to a prolongation of the contraction phase. Finally, loss of PRKAC activity inhibits phosphodiesterase-4 (PDE4D) and reduces phosphorylation of the ADRB1 receptors (thereby diminishing arrestin-mediated signaling). The functional consequences of the latter two events are not clear to us. (d) Effect of acetylcholine on rhythmicity of the SA-pacemaker cells. The pacemaker potential (slope of the funny current) is greatly prolonged (nearly doubled when going from 100 to 50 beats/min) but the action potential is also less compact because of a slightly slower depolarization (Ca^{2+} current) and repolarization phase (K^+ current).

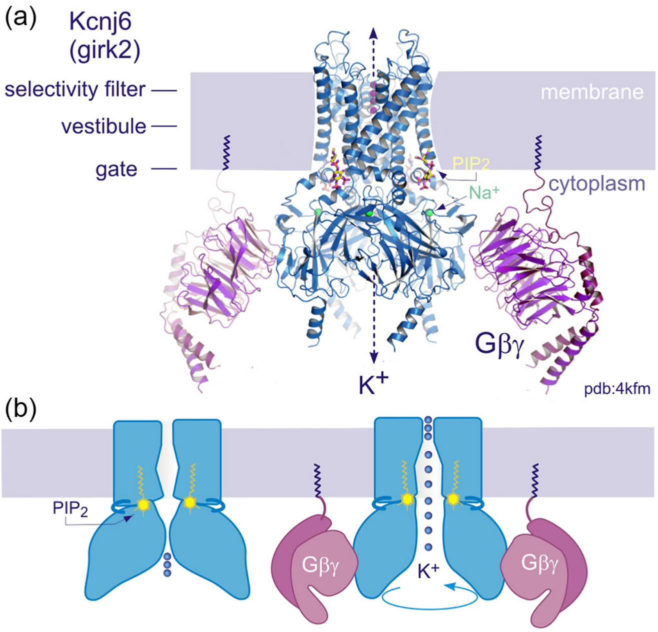


FIGURE 4-14 Molecular structure and gating mechanism of the mouse-brain inward rectifier channel complexed with Gβγ. (a) Realistic representation of the four identical subunits that constitute the mouse Kcnj6 (girk2) channel. The transmembrane segment of the channel is composed of a selectivity filter, a vestibule, and a gate. The proteins bind phosphatidyl-4,5-inositol bisphosphate and Na^+. The cytosolic segments bind Gβγ. (b) Inward rectifier channels undergo sequential activation, in which Na^+, PIP2, and Gβγ play a role. Gβγ causes a slight rotation of the cytosolic segments, relative to each other, and this causes the widening of the gate. PIP2 plays the necessary role of tightening the interaction between the transmembrane and cytosolic segment so that rotation is effectively translated in splaying of the helices surrounding the gate. *Schematic image adapted from Hansen et al. (2011). Pdb: 4kfm.*

physical activity, and other major cardiovascular risk factors, although the combination of smoking and having a high resting rate did elevate mortality slightly compared to nonsmokers with the same resting rate (Jensen et al., 2013).

We speak of inward-rectifier potassium channels and we show outward currents; that's rather confusing. The term rectification originates from an empirical description of ionic currents of channels that change conductance with a change of transmembrane voltage. The change in conductance may be the consequence of voltage-dependent channel gating (open/close) or a genuine voltage dependence of the open-channel current. Most channels have asymmetry of the conductance pathway and in the case of the potassium channels in question, the inward flow is greater

continued

at any given driving force (voltage) than the outward flow for the opposite driving force. That's why they were named inward-rectifier channels. Some inward-rectifier channels are very strict, they only allow K^+-entry, which may occasionally occur when other ions hyperpolarize the membrane potential below the K^+ resting potential or when extracellular K^+ is elevated (as in urine for instance). The KCNJ3 bound to Gβγ facilitates both inward and outward K^+-current but under the physiological conditions described here we only deal with outward flow (Nichols, 1997).

PHOSPHODIESTERASES

A superfamily

An essential condition for control of rhythmicity by acetylcholine is the existence of an enzyme that removes cAMP. Sutherland, who discovered the first second messenger, also for the first time described the enzyme that removes cAMP and which he named phosphodiesterase (PDE) (Butcher and Sutherland, 1962). A PDE breaks the cyclic bond of cyclic nucleotides, turning adenosine 3′,5′-cyclic monophosphate, or guanosine 3′5′-cyclic monophosphate, into adenosine 5′-monophosphate (5′-AMP) or 5′-GMP. Eleven families have been identified, PDE1–11, classified by their gene composition, their biochemical properties, mode of regulation, and sensitivity to pharmacological agents (Figure 4–15; Beavo, 1995; Lugnier, 2006; Bender and Beavo, 2006). Each family is composed of one or more members, they themselves being expressed in different splice variants.

All PDEs are composed of two segments, a regulatory and catalytic segment. The catalytic segment comprises 270–350 amino acids, and is common to all, whereas the regulatory segment varies per family. All members are expressed as homodimers with the exception of PDE6 which exists as homo- or heterodimer. Moreover, both types of dimers are bound to additional regulatory subunits (γ or δ). Regulation of activity affects Vmax, which may change 10-fold, and not affinity for the substrate (Bender and Beavo, 2006). Based on substrate specificity, the superfamily can be divided into three groups: the dual specificity, cAMP-specific, and cGMP-specific (Cancedo, 2013). Expression is nearly ubiquitous with the exception of PDE6 which is limited to the eye. We distinguish four regulation mechanisms; binding of Ca^{2+}/calmodulin (PDE1), binding of cGMP (PDE2, -5, and -6) or cAMP (PDE10A) to allosteric sites (GAF domain), phosphorylation by PRKAC (PKA), PRKC (PKC), AKT (PKB), PRKG (PKG), MAPK (ERK), or MAPKAPK2 (Lugnier, 2006). For members of PDE6, activation occurs through transducin (Gαt), which acts downstream of rhodopsin (the light receptor). It displaces the inhibitory subunits γ or δ thereby allowing access to substrate. Lastly, we mention that members of the PDE4 family bind to A-kinase-anchoring proteins and participate in signaling complexes that comprise protein kinase A (PRKAC), phosphatases, and their substrates (return to Chapter 3, Figure 3-22).

PDEs as targets for therapeutic purposes

PDEs are prime targets for pharmacological intervention because they interfere with both cAMP- and cGMP-controlled signaling pathways. They are preferred over nucleotide cyclases because their catalytic activity is at least a magnitude higher and as a consequence inhibition may have more impact (Nikolaev et al., 2005; Bender and Beavo, 2006). Immediately after the discovery of PDE activity, it was found that caffeine was an effective inhibitor. Theophylline, a caffeine analog also known as 1,3-dimethylxanthine, was the first nonselective inhibitor to be used for experimental purposes in 1962 (Butcher and Sutherland, 1962). One decade later, a new xanthine analog, 1-methyl-3-isobutylxanthine (IBMX) came to light which was 100-fold more potent than theophylline, and inhibiting PDE1 to -5. New, more or less specific inhibitors have since been developed (Figure 4-15) and these are currently being tested for therapeutic efficacy (or already employed) in areas of chronic immune disorders (such as asthma, psoriasis, or arthritis), heart failure, pulmonary hypertension, and erectile dysfunction (see section dealing with nitric oxide at the end of this chapter).

Substrate specificity and catalytic mechanism

PDEs employ a hydrophobic clamp (side chains of phenylalanine, isoleucine, and leucine) as well as a set of hydrogen bonds to bind the base (adenine or guanine) of the cyclic nucleotide. Among the hydrogen bonds, those employed by glutamine play a key role because it determine whether or not the enzyme is dual-specific. Glutamine can make two hydrogen bonds with either cAMP or cGMP but this occurs in two different orientations (Figure 4-16). For monospecific PDEs, the orientation of glutamine is stabilized through interaction with an adjacent amino acid. In the case of PDE4D, tyrosine (Y329) prevents rotation and as a consequence glutamine (Q319) can only engage hydrogen bonds with cAMP (and not with cGMP). For PDE5A, glutamine (Q775) anchors Q817 in an opposite orientation and, as a consequence, it can only engage hydrogen bonds with cGMP. PDE1B, a dual-specific PDE, lacks supporting amino acids and the orientation of the essential glutamine side chain is not constrained. It freely adapts to the incoming cyclic nucleotide. Concerning the catalytic activity of dual-specific PDEs, both the Km (affinity) and Vmax (velocity) may vary between the two nucleotides (Bender and Beavo, 2006).

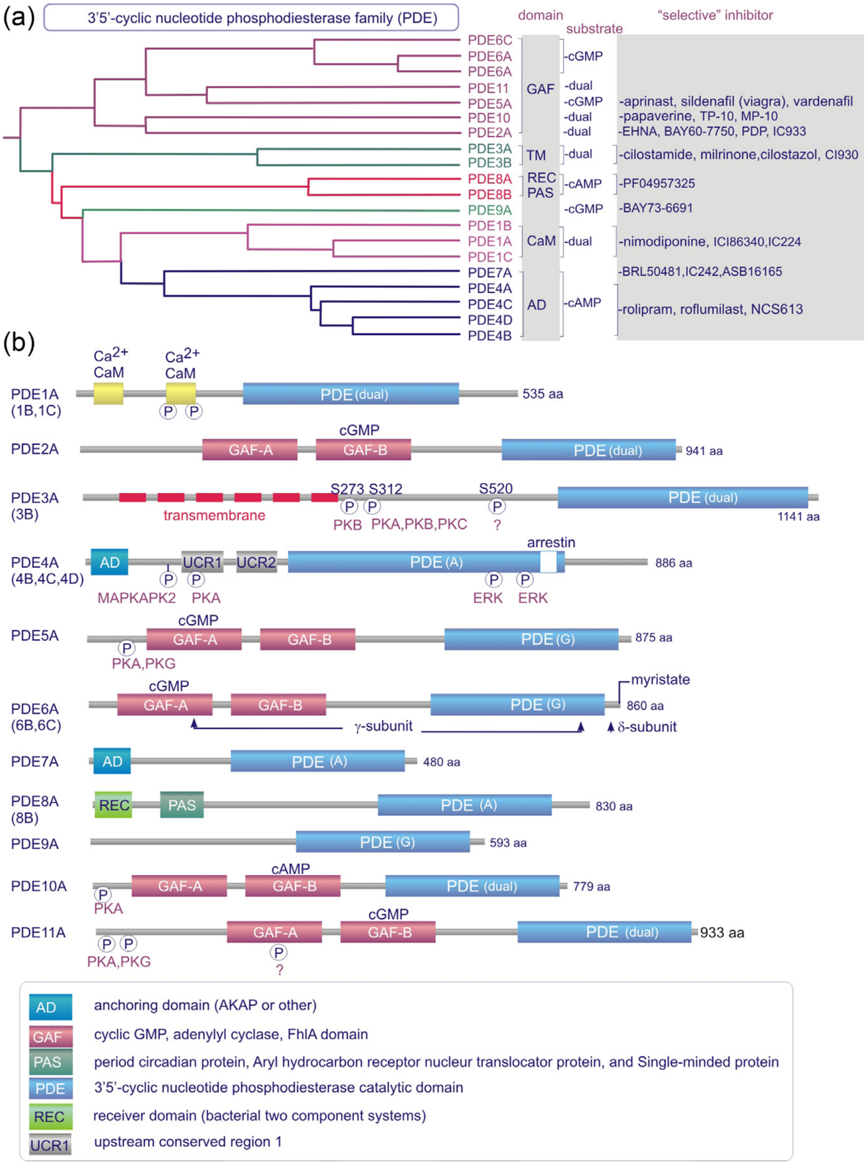


FIGURE 4-15 Phylogenetic relationship and domain architecture of members of the human phosphodiesterase (PDE) superfamily. (a) The superfamily is subdivided into five groups on the basis of the identity of the additional noncatalytic domains (GAF, TM (transmembrane), REC/PAS, CaM (calmodulin binding), and AD). As you will notice, substrate specificity varies within the GAF group (either dual specific or cGMP). PDEs are thought after targets for pharmacological intervention in the field of immunity, cardiovascular diseases, and erectile dysfunction, hence the development of numerous inhibitors. (b) The domain architecture of the 11 families illustrates the shared PDE-domain and the unique upstream regulatory domains (CaM, GAF, UCR, REC/PAS) or "subcellular localization" (TM, AD). It is important to distinguish between substrate specificity (cGMP and/or cAMP) and allosteric regulation by the same cyclic nucleotides. As an example, PDE11A is a dual specific PDE whose activity is stimulated by cGMP.

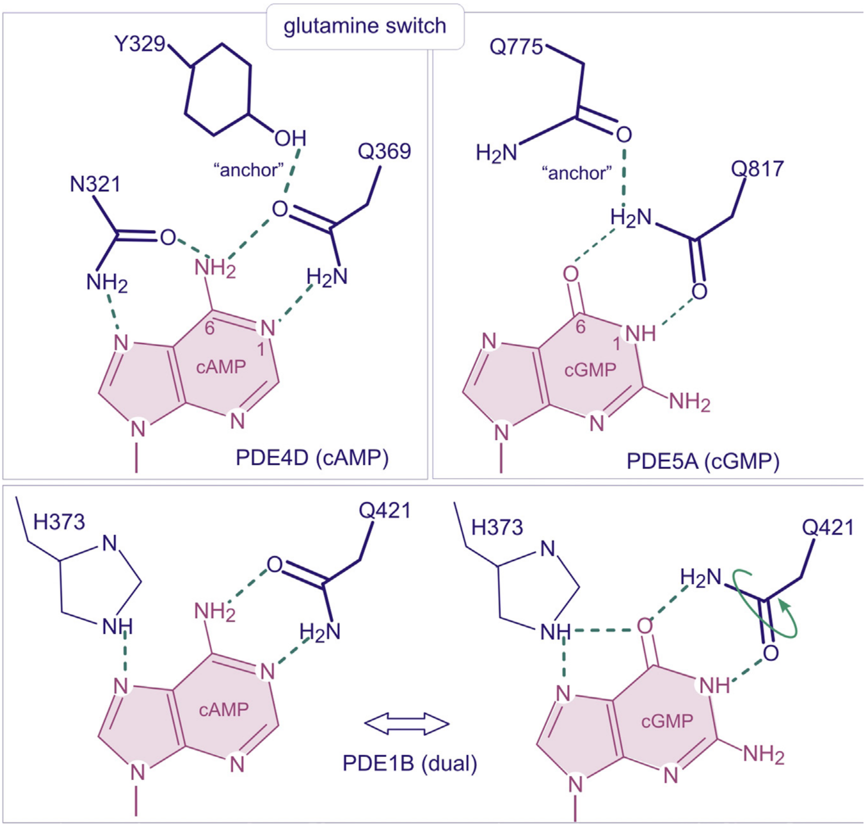


FIGURE 4-16 Phosphodiesterases (PDEs) bind the cyclic nucleotide base (adenine or guanine) with a hydrophobic clamp (side chains of phenylalanine, isoleucine, and leucine, not shown) and with a set of hydrogen bonds. Glutamine (Q) plays an essential role in the coordination of either NH(1) and O(6), the case of adenine, or N(1) and NH2 (6), the case of guanine. In monospecific PDEs, the orientation of the side chain of glutamine is anchored, through a hydrogen bond, by an adjacent tyrosine (Y329, for PDE4D) or by an adjacent glutamine (Q775, for PDE5A). As a consequence, PDE4D can employ two hydrogen bonds with cAMP but none with cGMP, whereas PDE5A can employ two hydrogen bonds with cGMP but none with cAMP. In other words, binding is selective. In the case of dual specificity PDEs, the position of glutamine side chain is not stabilized and, through rotation, it adapts its position to the entering cyclic nucleotide. *Image adapted from Zhang et al. (2004).*

The catalytic residue of PDEs is a protonated histidine (which occurs in a highly conserved YH*N motif). It positions near the 3′-ester bond of the cyclic nucleotide. The reaction starts with a nucleophilic attack of water, which has been rendered nucleophilic (OH^-) because of two metal ions (Zn^{2+} and Mg^{2+}). These ions also coordinate and neutralize the negative charge of two oxygen atoms of the phosphate (Figure 4-17). The transfer of the electron causes the hydroxide to bind to the phosphor (transient pentameric state). The extra electron next breaks the 3′ bond between the

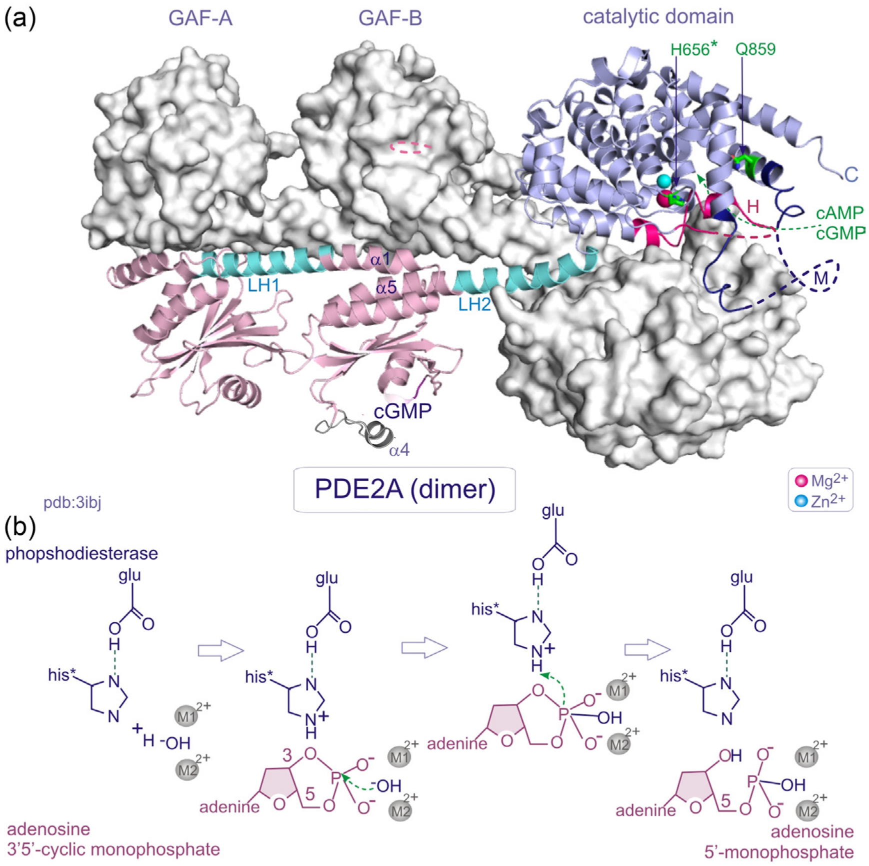


FIGURE 4-17 Molecular structure of the PDE2A dimer and mode of action of phosphodiesterases. (a) Human PDE2A occurs as a homodimer (one shown in gray surface, the other in cartoon). Each possesses two regulatory GAF domains (A and B) and a catalytic domain (C-terminal). Linker helices (LH1 and -2) connect the different domains. PDE2A hydrolyzes the 3′ bond of both cAMP and cGMP (dual-specific enzyme). The GAF-B domain binds cGMP which acts as an allosteric stimulus (enhancing the Vmax sixfold). The α4-helix is grafted on the structure (not visible in the electron density map). The catalytic site, containing an Mg^{2+} and a Zn^{2+} ion, is indicated by the highly conserved glutamine (Q859) and the catalytically important electrophile histidine (H656) (in green). H and M represent the H- and M-loop respectively. The dimer is in its inactive conformation, access of substrate is blocked. Pdb: 3ibjb. (b) Breaking of the 3′ ester bond occurs through a nucleophilic attack by water, rendered nucleophile (OH$^-$) by the presence of two metal ions. The protonated histidine (H656 in PDE2A) serves as the electrophile and causes the bond breakage at the 3′-position. *Catalytic scheme adapted from Liu et al. (2008). Pdb: 3ibj.*

ribose and the phosphor and engages the H$^+$ from histidine (the electrophile) to form the ribose-3′OH (Liu et al., 2008).

Regulatory domains affect enzyme activity but it is not clear yet how. PDEs form parallel dimers, in which both proteins occur in the same orientation (Figure 4-17). The crystallographic structure of the near-full-length human PDE2A reveals that, in its inactive state, the catalytic domains contact each other and this hinders substrate binding. In particular, the orientation of the M-loop seems to make access impossible. Binding of cGMP to the allosteric site on the GAF-B domain causes a change in the relative position of the α1- and α5-helices. It is proposed that this causes the two catalytic domains to swing out relative to each other. As a result, the M-loop moves outward and renders the catalytic site accessible (Pandit et al., 2009). More crystallographic studies are needed to substantiate this proposed mode of action.

Homologous and heterologous pathway control through PDEs

Regulation of activity of PDEs by cGMP, PRKAC, or PRKG creates the opportunity for negative feedback and pathway cross talk. Negative feedback occurs in two fashions: phosphorylation by either the cGMP- or cAMP-dependent protein kinase (PRKG or PRKAC) and through activation by cGMP. What it means is that a pathway not only can restrain its own extent (PRKAC activates PDE4A and increases the breakdown of cAMP) but also of another pathway (cGMP activates PDE2A and limits both the cGMP- and cAMP-signaling events). Heterologous control is perhaps best exemplified in adrenal glomerulosa cells, located in the superficial layer of adrenal cortex, in which PDE2A is highly expressed and mediates the inhibitory effect of atrial natriuretic peptide (ANP) on aldosterone secretion (a steroid that regulates AQP expression in the collecting tubules of the kidney). ANP is released by atrial myocytes in response to stretch induced by increased afterload. It causes a rise in cGMP in glomerulosa cells because it is a transmembrane receptor with an intracellular guanylyl cyclase domain. cGMP diffuses into the cytosol, binds the GAF-B domain of PDE2A and through stimulation of the enzyme it lowers the level of adrenocorticotropic hormone-induced production of cAMP. Loss of cAMP causes the loss of aldosterone secretion (MacFarland et al., 1991; Nikolaev et al., 2005; Figure 4-18). Less aldosterone translates into a reduced retention of water in the kidney and thus a lowering blood volume, hence a reduction in blood pressure.

Web resources

- PDE HUGO http://www.genenames.org/genefamilies/PDE
- For more information about phosphodiesterases and pharmacological intervention in disease, Lugnier C. PDE inhibitors: a new approach to treat metabolic syndrome? Current Opinion in Pharmacology 2011; 11:698–706.

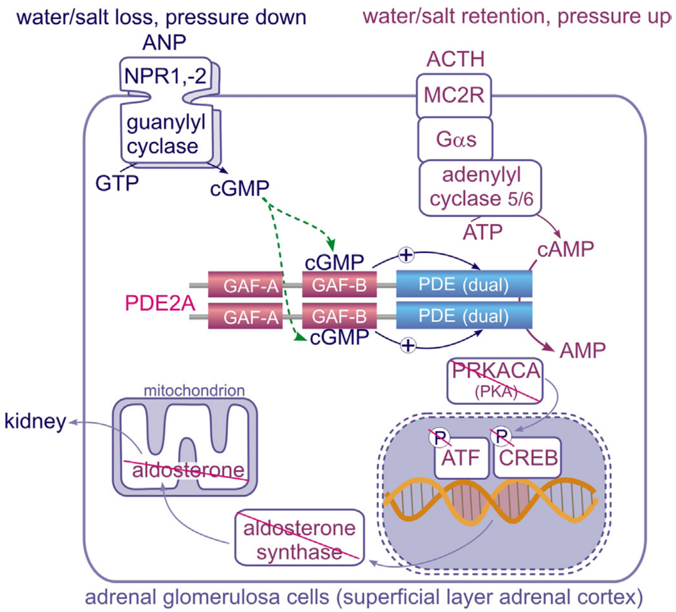


FIGURE 4-18 Heterologous pathway control through the dual-specificity phosphodiesterase-2A. The production of aldosterone, a steroid produced by adrenal glomerulosa cells, is stimulated by ACTH through the cAMP/PRKACA pathway. This pathway phosphorylates and activates the transcription factors ATF and CREB, which drive expression of aldosterone synthase, a key enzyme in the synthesis pathway. Aldosterone is released into the blood and causes salt and water retention in the kidney. The atrial natriuretic peptide (ANP) activates the guanylyl cyclase activity that is carried by its receptors (occurring as a dimer). The rise in cGMP enhances PDE2A activity through allosteric regulation via the GAF-B domain. This, in turn, causes a rapid and efficient breakdown of cAMP and deprives the ACTH pathway from its second messenger and aldosterone release will grind to a halt. Of note, cGMP will also be removed by PDE2A, example of autologous control. ACTH: adrenocorticotropic hormone.

ACETYLCHOLINE, ACTING ON THE M3 RECEPTOR, CAUSES AIRWAY CONSTRICTION AND MUCUS SECRETION

Airway constriction

Acetylcholine muscarinic M3 receptors (CHRM3) of the airway, expressed in the trachea and bronchi, have long been recognized to cause vagally induced airway smooth muscle contraction and mucus secretion, two characteristics of asthma. Indeed, initially asthma was considered primarily a nervous disease, asthma nervosum, as reported by the Dutch physiologist and Nobel Laureate Willem Einthoven back in 1892, even although a contribution of inflammation was recognized (Einthoven, 1892; Meurs et al., 2013). More than 100 years later, the question about what causes asthma, being primarily the consequence of vagal overstimulation or allergic inflammation, is still not resolved. It probably is the consequence of both (Galli et al., 2008; Lewis et al., 2006). The M3 receptor is principally coupled to the Gq/11 subfamily of G-proteins of which Gαq is expressed in airway smooth muscle. The signaling pathway resembles the one described for the α1-adrenoceptor-mediated vasoconstriction in visceral arteries/arterioles (return to Chapter 3, Figure 3-28). Activation of the G-protein leads to Gαq-mediated stimulation of phospholipase-Cβ, giving rise to the conversion of PIP_2 into the two second messengers inositol-1,4,5-trisphosphate (IP_3) and DAG (return to Chapter 3, Figure 3-15). IP_3 diffuses into the cytosol and binds its receptor (ITPR1) on the sarcoplasmic reticulum, thereby, augmenting the release of Ca^{2+}. The rise in intracellular free Ca^{2+} next enhances conductivity of neighboring ryanodine receptors (RYR2) and together they generate a transient Ca^{2+}-wave. Both the IP_3- and ryanodine-receptor have a biphasic conductance pattern and this is explained by the presence of two Ca^{2+} sites: a high affinity which causes channel opening and a low affinity which causes channel closure. So initially Ca^{2+} causes release of Ca^{2+} until a certain concentration where the conductance is blocked. Binding of IP_3 reduces the inhibitory effect and the IP_3-receptor channels remain open for a longer time thus giving rise to a genuine Ca^{2+} wave which may last for seconds (Mak et al., 1998). Some 25 waves/min have been detected in lung slices of mice treated with methacholine (nonselective muscarinic receptor agonist which is used in the diagnosis of bronchial hyperreactivity, a hallmark of asthma and chronic obstructive pulmonary disease (COPD)) (Bai and Sanderson, 2006). The frequency of the Ca^{2+} oscillations determines the contraction state of the smooth muscle cell (Perez and Sanderson, 2005). The above-described oscillations pursue in the absence of IP_3 but they are limited to short range, low-frequency sparks. These only cause contraction twitches (Miyakawa et al., 2001).

Intracellular free Ca^{2+} binds calmodulin and the complex binds and activates myosin light-chain kinase (MYLK or smMLCK) (return to Chapter 3, Figures 3-28–30 for details). MYLK phosphorylates the regulatory myosin light chain (MYL12B) on Ser19 and this allows the actin-myosin cross-bridge cycle to operate. In parallel, both Ca^{2+} and DAG recruit protein kinase C (PRKC) to the membrane. The active protein kinase phosphorylates an inhibitory regulator of myosin-light chain phosphatase named CPI-17 (or PPP1R14A) on thr-38 (Li et al., 1998). Its phosphorylation augments the affinity for the catalytic subunit of the myosin light-chain phosphatase, that is, inhibitor and phosphatase get tightly bound. The subsequent loss of phosphatase activity makes the system extremely sensitive to Ca^{2+} (because of a lack of opposition). The precise identity of the protein kinase-C enzyme is not yet revealed (at least seven different members of the PRKC-family are sensitive to DAG). As we have described for the α1-adrenoceptor, some M3 receptors interact with Gα12, a G-protein subunit which activates the RGS domain-containing guanine-exchange protein RhoGEF. The exchange of GDP for GTP on RhoA causes interaction with- and activation of Rho-kinase, ROCK1, and this too results in phosphorylation of CPI-17 (Figure 4-19).

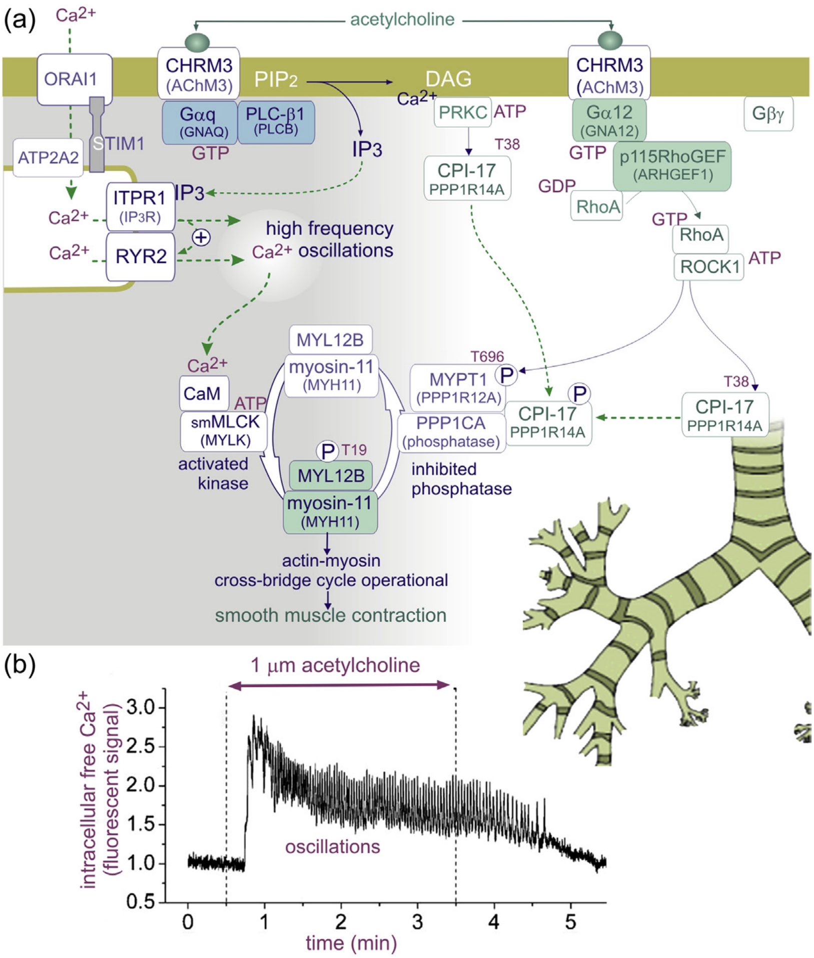


FIGURE 4-19 Bronchial smooth muscle contraction induced by acetylcholine acting on M3 receptors. (a) Bronchial smooth muscle cells express the acetylcholine M3 receptor which is predominantly coupled to Gαq. The ensuing PLC-b1 activation leads to the production of two second messengers, the cytosolic inositol-1,4,5-trisphosphate (IP_3) and the membrane bound diacylglycerol (DAG). IP_3 binds its receptor on the sarcoplasmic reticulum and enhances the release of Ca^{2+}. This in turn enhances Ca^{2+} release through the ryanodine receptor-2 (RYR2) resulting in strong Ca^{2+}-oscillations. Ca^{2+} binds calmodulin and together they stimulate the smooth muscle myosin light-chain kinase (MYLK). Subsequent phosphorylation of the myosin regulatory subunit (MYL12B) on thr-19 allows the actin–myosin cross-bridge cycle to operate and the muscle contracts. DAG and Ca^{2+} recruit a phospholipid-dependent protein kinase to the membrane (PRKC or PKC) and its activation causes phosphorylation of CPI-17 (or PPP1R14A) on thr-38. In parallel, acetylcholine activates Gα12 which leads to the activation of a guanine-exchange protein for RhoA (ARHGEF1). The subsequent activation of the monomeric GTPase RhoA gives rise to the activation of a G-protein-stimulated protein kinase ROCK1 (Rho-associated-coiled-coil containing- protein kinase-1). ROCK1 plays an essential role in sensitizing the smooth muscle for Ca^{2+} in two ways. First it phosphorylates the regulatory subunit of the myosin light-chain phosphatase (MYPT1 or PPP1R12A) on thr-969. This causes the dissociation between substrate and phosphatase. It also phosphorylates, like PRKC, the inhibitory subunit CPI-17. Phosphorylated CPI-17 tightly binds the myosin light-chain phosphatase and reinforces its inhibition. The myosin regulatory-subunit remains fully phosphorylated because of a lack of phosphatase opposition. (b) Ca^{2+}-oscillations caused by the addition of acetylcholine to smooth muscle cells of bronchi. *Image obtained with permission from Perez and Sanderson (2005)).*

ROCK1 also phosphorylates MYP1 (or PPP1R12A) on thr-969 and this separates the phosphatase from its substrate, the regulatory subunit of smooth muscle myosin (MYL12B).

Other Ca^{2+}-release channels operating in smooth muscle

The sequence we have presented is a simplified version of what really happens because there are at least three more Ca^{2+} channels that operate in smooth muscle. First, we mention the short transient receptor potential channel-3 (TRPC3), a calcium-permeant cation channel (Wang and Zheng, 2011). It belongs to the large family of six-membrane spanning transient receptor potential (TRP) channels that assemble as tetramers to form cation-permeable pores. TRP channels are best known for their role in sensory systems: detecting heat and cold, taste, pain (Clapham, 2003) and they play a role in guidance of axon outgrowth (Li et al., 2005). In airway smooth muscle, TRPC3 senses IP_3-mediated Ca^{2+} release from the sarcoplasmic reticulum and thus qualifies as a store-operated channel. It forms a complex with the ligand-bound IP_3-receptor and this leads to increased ion-conductance of extracellular Ca^{2+} and Na^+. The ensuing modest membrane depolarization engages the second channel, the long-lasting (L-type) voltage-sensitive Ca^{2+} channel (CACNA1C or Cav1.2) which contributes to an enhanced Ca^{2+} entry (Narayanam et al., 2012). One study has shown that this channel is a target of PRKC and phosphorylation increases its conductivity (Mukherjee et al., 2013). Finally, IP_3-mediated Ca^{2+} depletion of the sarcoplasmic reticulum causes the opening of the third channel, the store-operated Ca^{2+} channel ORAI1. It too associates with the sarcoplasmic reticulum through its interaction with the Ca^{2+}-sensor STIM1 (Peel et al., 2008). The relative contribution of all these Ca^{2+} currents in smooth muscle contraction is hard to assess, as are the operating feed-forward and -backward control mechanisms (Tao et al., 2013). The constant inward flow of Ca^{2+} through these channels is counteracted by Ca^{2+}-ATPases, located both in the plasma membrane (ATP2B2) and in the sarcoplasmic reticulum (ATP2A2 or SERCA2). We will return more extensively to the regulation of Ca^{2+} waves in the Chapter 6 about “intracellular Ca^{2+} and its effectors.”

Removal of second messengers

The signal induced by inositol-1,4,5-trisphosphate (IP_3) can be terminated in two ways. One concerns an inositol trisphosphate 3-kinase (ITPKA, -B, or -C), giving rise to the generation of inositol-1,3,4,5-tetrakisphosphate (IP_4), and the other an inositol-1,4,5-trisphosphate

5-phosphatase (INPP5A and -5B), giving rise to the generation of inositol-1,4-bisphosphate (Xia and Yang, 2005; Erneux et al., 1998). Both metabolites are further processed and are then reutilized for the production of phosphatidyl-inositol lipids. We note that IP_4 is not just an intermediate metabolite. In B-lymphocytes, it has been reported to play a role in regulating the immune response by acting as a negative-feedback second messenger. It inhibits store-operated Ca^{2+} channels as well as the IP_3R (Sauer and Cooke, 2010).

DAG is removed by a lipid kinase (DGK), leading to the formation of phosphatidate (phosphatidic acid). The enzyme forms a family with 10 members, denoted DGK-α to -κ. It is not clear which one(s) operates in airway smooth muscle. Phosphatidate is further processed by phosphatidate cytidylyltransferase, giving rise to CDP-DAG, which is also reutilized for the production of phosphatidyl-inositol lipids. Like for IP_4, phosphatidate is not just a metabolic intermediate, it interacts with and modifies activity of a number of protein kinases and phosphatases (among which mTOR, Raf1, PPP1C, and SHP1). DAG kinase has been studied extensively in T-lymphocytes and, like IP_4, it plays a role in keeping in check the immune response by promoting self-tolerance and preventing T-cell hyperactivation (Krishna and Zhong, 2013).

Web resources

KEGG, Kyoto Encyclopedia of Genes and Genomes.

- Phosphatidylinositol-signaling system map04070 (http://www.genome.jp/dbget-bin/www_bget?pathway:map04070)

Mucus production

The M3-mediated signaling pathway that causes smooth muscle contraction also operates in airway epithelial cells. In the context of mucus production, both serous cells in the submucosal glands and goblet cells of the airway lining are concerned (Figure 4-20). Mucus production is the consequence of a simultaneous extrusion of salt (NaCl) and secretion of mucins (highly glycosylated proteins of which Mucin2, -5B, -7, -8, and -19 are expressed in the lung). Water follows passively, resulting in a moderately viscous fluid that covers the epithelial lining of the airway (roughly 10-μm thick and named airway surface liquid) (Williams et al., 2006). A constant flow of mucus, brought about by beating cilia of the epithelial cells, and going from bronchi to the esophagus, is essential for the removal of dust and microbes of all sorts. However, excessive production

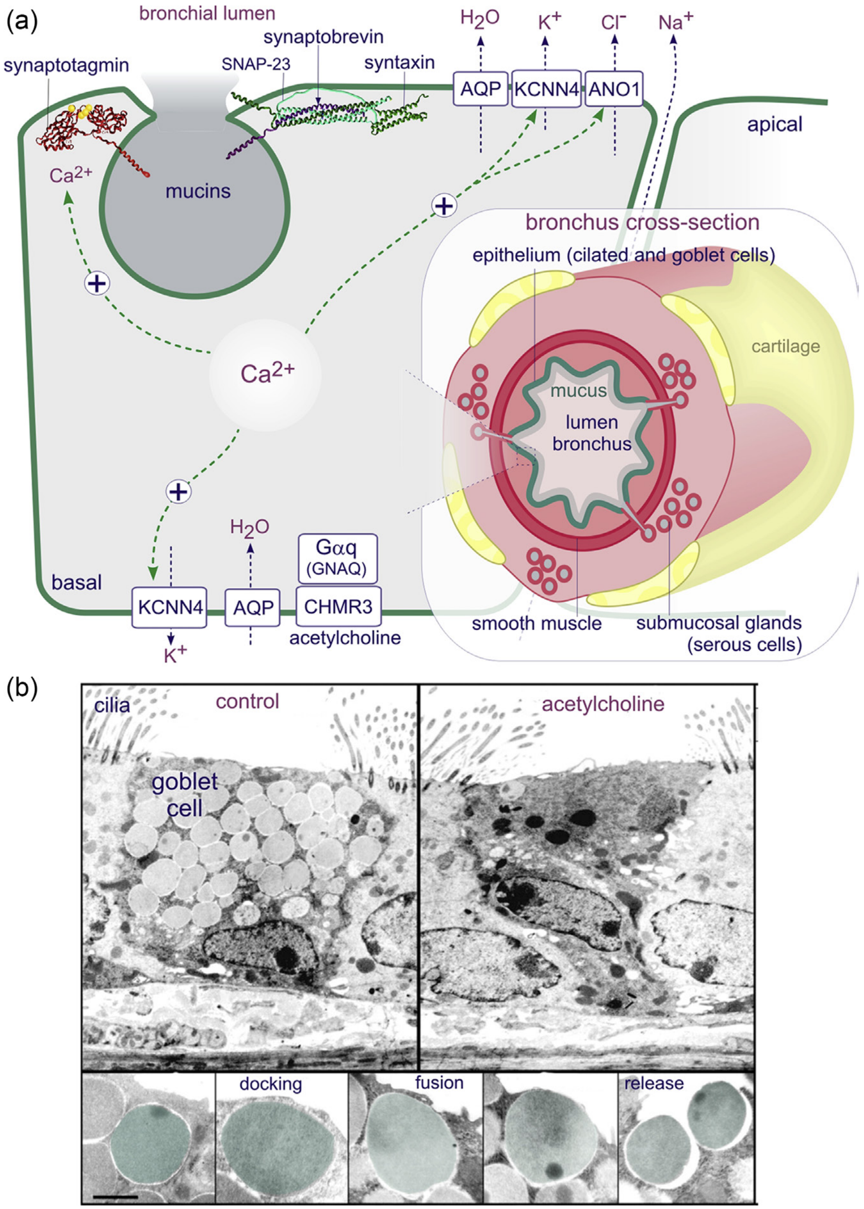


FIGURE 4–20 Bronchial mucus production in response to acetylcholine. (a) Intracellular free Ca^{2+}, which level increases in response to acetylcholine, binds to synaptotagmin and this leads to fusion of mucin-charged vesicles with the plasma membrane. Ca^{2+} also enhances conductance of the KCNN4-channel (K^+) and the ANO1-channel (Cl^-). Na^+ and H_2O passively follow the ion movements. Together, ions, mucins, and water, constitute bronchial mucus. (b) Electron microscopy images of bronchial epithelial goblet cells discharging its secretory vesicles after stimulation with acetylcholine. The insert shows the different phases of exocytosis. *Image modified from* Williams et al. (2006).

causes occlusion of small- and medium-sized airways and this gives rise to breathlessness (dyspnea) (Dunnill, 1960).

Acetylcholine brings about an increased mucus secretion through the rise in intracellular free Ca^{2+}. It binds synaptotagmin, a Ca^{2+}-binding component of the membrane fusion machinery that comprises SNAP-23, synaptobrevin and syntaxin (members of the SNARE family), and this leads to membrane fusion and release of the vesicle content (process of exocytosis). Intracellular free Ca^{2+} also activates the outward flow of K^+ (through the Ca^{2+}-sensitive KCNN4 channel situated at the basal and apical side), which, because of the ensuing hyperpolarization, facilitates the release of Cl^-, which occurs through the Ca^{2+}-sensitive ANO1 or CLCA1 channel, situated at the apical side (lumen of the airway). Chloride release exceeds that of K^+ at the apical side and this leads to the arrival of Na^+ (charge compensation). Na^+ diffuses predominantly through the intercellular space. Salt and glycoproteins subsequently capture water, which enters the airway lumen via aquaporins (AQP5 at the basal side and AQP1, -3, -4 at the apical side) (Frizzell and Hanrahan, 2012).

Chloride transport also involves the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel which dysfunctions in cystic fibrosis. It is expressed at the basal side of the airway epithelium and, in the context of enhanced mucus secretion, plays a role in letting Cl^- enter the epithelial cell so that it can enter the airway lumen. Loss of function of CFTR causes a reduced salt content of the mucus and thus a reduction of water content. The sticky mucus prevents clearance of the lungs, the ciliary movement is hampered, and this renders them susceptible to opportunistic infections (which is the main cause of death of people who carry the defect) (Frizzell and Hanrahan, 2012).

Web resources

KEGG, Kyoto Encyclopedia of Genes and Genomes.

- Asthma, map05310 (http://www.genome.jp/kegg-bin/show_pathway?map05310)
- Mucus secretion, map04970 (http://www.genome.jp/dbget-bin/www_bget?pathway:map04970)

Transporter classification database operated by the Saier Lab Bioinformatics Group http://www.tcdb.org/

Other roles of acetylcholine in asthma

Recent studies have revealed that acetylcholine also exerts proinflammatory, pro-proliferative, and pro-fibrotic actions in the airways, which may involve muscarinic receptor-stimulation on mesenchymal, epithelial, and inflammatory cells. Moreover, acetylcholine in the lungs may not only be derived from vagal nerves, but also from non-neuronal cells, including epithelial and inflammatory cells. Cholinergic contractile tone is increased by airway inflammation associated with asthma and COPD, resulting from exaggerated acetylcholine release as well as increased expression of contraction-related proteins in airway smooth muscle. Moreover, muscarinic receptor stimulation promotes proliferation of airway smooth muscle cells as well as fibroblasts, and regulates cytokine, chemokine, and extracellular matrix production by these cells, which may contribute to airway smooth muscle growth, airway fibrosis, and inflammation (Meurs et al., 2013).

β2-adrenoceptor agonists provide relief but the future may be for acetylcholine antagonists or PDE4 inhibitors

The relaxation of airway smooth muscle tone can be obtained both by a reduction of the frequency of the Ca^{2+} oscillations and/or by a reduction in the Ca^{2+} sensitivity. Adrenoceptor β2-agonists do both and that is why they provide instant relief when inhaled in the lungs during episodes of asthma. Examples of short-acting agents are salbutamol and terbutaline, and of long-acting agents, salmeterol and formoterol (return to Chapter 3, Figure 3-8 for structure of salbutamol). β2-adrenoceptors are coupled to Gαs and they cause activation of adenylyl cyclase, the production of cAMP, and subsequent activation of protein kinase A (PRKACA) and the guanine exchange factor EPAC. We highlight three mechanisms by which cAMP causes smooth muscle relaxation.

With respect to Ca^{2+} oscillations, PRKACA phosphorylates the large conductance Ca^{2+}-sensitive K^+-channel (KCNMA1, also known as $K_{Ca}1.1$ or BK_{Ca}) and enhances its outward current (Koh et al., 1996). Enhanced outward flow of K^+ causes membrane hyperpolarization thereby shutting down the voltage-sensitive Ca^{2+}-channel (CACNA1C). This reduces the frequency of Ca^{2+} oscillations. Concerning Ca^{2+} sensitivity, PRKACA phosphorylates the monomeric GTPase RhoA, on Ser-188; and this tightens its interaction with RhoGDI (ARHGDIB) (Ellerbroek et al., 2003). GDI stands for guanine-dissociation inhibitor; it prevents the exchange of GDP for GTP as long as the two are bound. Inaccessible RhoA means the loss of activation of the kinase ROCK1 and thus less phosphorylation of the phosphatase inhibitor CPI-17 (PPP1R14A) and of MYPT1 (PPP1R12A). This frees the myosin light-chain phosphatase from inhibitory constraints and the balance shifts to dephosphorylation of the regulatory myosin light-chain (MYL12B). The actin–myosin cross-bridge cycles diminish and this causes the muscle to relax. In parallel, PRKACA phosphorylates telokin on serine (pSer13). This causes it to bind to the regulatory subunit (MYPT) of myosin light-chain phosphatase. Binding of telokin also relieves all inhibitory constraints and also shifts the kinase/phosphatase balance in favor of phosphatase activity (Khromov et al., 2012).

Another way to prevent activation of RhoA/ROCK1 involves another effector of cAMP, namely the guanine-exchange factor directly activated by cAMP (RAPGEF3 or EPAC1). This is a nucleotide-exchange factor for the monomeric GTPase Rap1 (Ras-related protein). Cyclic AMP binds RAPGEF3 in a manner similar as it binds the regulatory subunit (RI or RII) of PRKACA. The binding causes a conformation change which allows RAPGEF3 to bind Rap1 and exchange its GDP for GTP. The activated Rap1 reduces the amount of activated Rho (RhoGTP) but the mechanism by which this occurs remains elusive (Zieba et al., 2011; Roscioni et al., 2011). The consequences are the same as described for phosphorylation of RhoA (Figure 4-21).

Telokin is in fact isoform-6 of myosin light-chain kinase (MYLK). It is produced by alternative initiation at met-1761 during the translation process (ribosome), giving rise to a truncated protein of 210 amino acids. Telokin is nothing more than the C-terminal segment of MYLK containing a calmodulin-binding domain and an immunoglobulin-like C2-type9 domain. Phosphorylation on ser-13 means at position 1773 in the whole protein. How the phosphorylated protein relieves inhibitory constraints is not known.

Despite the immediate clinical benefit of β-agonists in asthma therapy, regular use may in fact promote harmful effects in the longer term. A number of studies link chronic application with adverse patient outcomes, such as waning of β2-agonist-mediated relaxation (tachyphylaxis), deterioration of asthma control, and premature death (compared to a control group) (Salpeter et al., 2006; Walker et al., 2011). Despite its widespread use, there still is a lack of mechanistic understanding of the physiological effects of chronic β2-adrenoceptor stimulation. Fingers are pointed to arrestins and inflammatory cytokines, such as TNFα, as being culprits of receptor desensitization (Deshpande et al., 2008; Vasudevan et al., 2011).

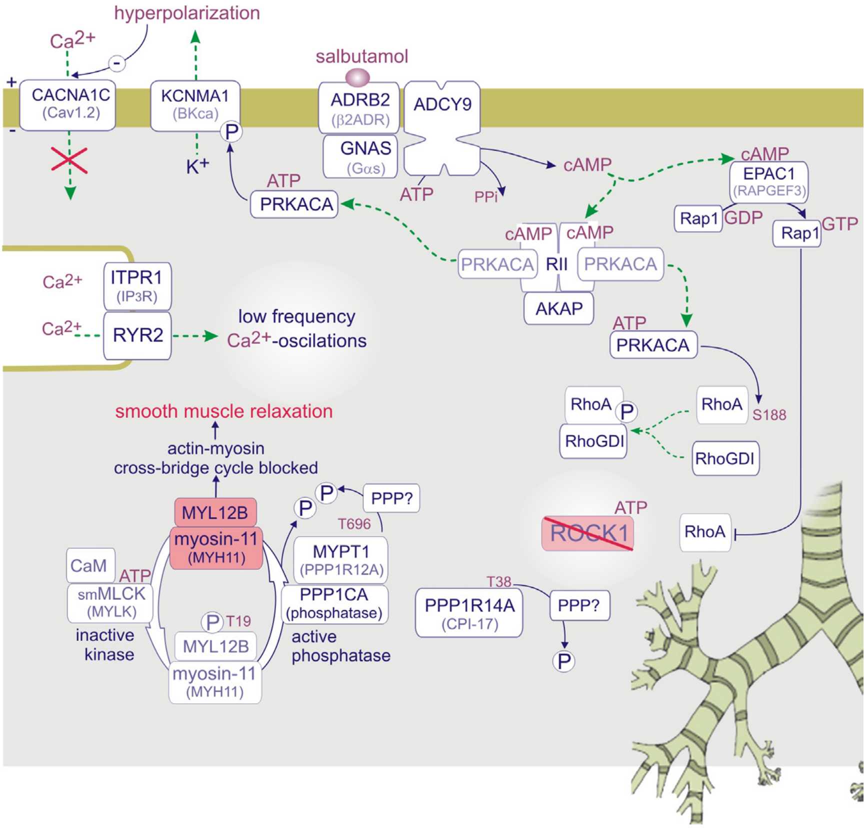


FIGURE 4-21 β2-agonist-mediated airway smooth muscle relaxation. Salbutamol, binding the β2-adrenoceptor, is an adrenergic agonist and, in the context of bronchial smooth muscle, acts as a functional antagonist of acetylcholine. It blocks smooth muscle contraction at a number of signaling events. The β2-adrenoceptor is coupled to Gas and leads to activation of adenylyl cyclase (ADCY9) and production of cAMP. Two effectors are concerned, the cAMP-dependent protein kinase (PRKACA) and the guanine-exchange protein activated by cAMP, EPAC (RAPGEF3). Activation of PRKACA is caused by its dissociation from a regulatory subunit (RII). One of the substrates is the Ca^{2+}-sensitive K^{+} large-conductance channel (KCNMA1) and its phosphorylation increases permeability. The ensuing hyperpolarization reduces Ca^{2+} influx through the voltage-sensitive CACNA1C channel and this reduces the frequency the Ca^{2+} oscillations. PRKCA also phosphorylates the monomeric G-protein RhoA and this enhances its affinity for RhoGDI. GDI stands for guanine dissociation inhibitor, which means that RhoA bound to RhoGDI is not accessible to guanine-exchange factors. The "depletion" of functional RhoA prevents activation of ROCK1, a protein kinase that plays a key role in holding in check myosin light-chain phosphatase activity. The other target of cAMP, EPAC1, causes the activation of the monomeric G-protein Rap1 and this, through unknown mechanism, blocks activation of RhoA. Serine/threonine phosphatases (indicated by PPP of unknown identity) remove the phosphates from MYPT1 and CPI-17, relieving PPP1CA from inhibitory constraints. Low-frequency Ca^{2+}-oscillations and reactivation of the myosin light-chain phosphatase (PPP1CA) result in smooth muscle relaxation and provides instant relief of breathlessness during an asthmatic episode.

An alternative to functional antagonism by β2-agonists is preventing acetylcholine binding to the M3 receptor (true receptor antagonism). A possible therapeutic role for "anticholinergics" gained new interest with recent evidence arising from the UPLIFT study (Understanding Potential Long-term Impacts on Function with Tiotropium). Tiotropium (trade names Spiriva or Tiova) is derived from atropine and is a nonselective muscarinic-receptor antagonist. It improves lung function and reduces exacerbation frequency in patients with COPD (return to Figure 4-10 for an image of tiotropium bound to the M3 receptor) (Tashkin et al., 2008). Yet, another alternative is the employment of highly selective PDE4 inhibitors which not only have relaxation effects, by raising cAMP levels, but also anti-inflammatory qualities. Currently, the existing inhibitors suffer from significant adverse effects such as diarrhea and nausea and, for the time, a combination of anti-inflammatory steroids (such as prednisolone) and β2-agonists remain the mainstay in asthma treatment (Beghe et al., 2013).

ACETYLCHOLINE AND THE INDUCTION OF NITRIC OXIDE, A POTENT VASODILATOR

Endothelial cell-derived relaxing factor

The precise role of acetylcholine in the regulation of blood pressure has been puzzling for some time for numerous reasons. First of all, a distinction has to be made between its action on the heart (reducing rate and force), the kidneys (increasing diuresis), and the vasculature (vasodilatation/contraction). Then, high doses penetrate the autonomic ganglia of the sympathetic trunc and stimulate the sympathetic system (causing the release of noradrenaline) (return to Chapter 3, Figure 3-2). Studies with arterial strips (ex vivo) would circumvent all this but here too nothing seemed straightforward at the start. In the first studies, acetylcholine was reported to contract (Furchgott and Bhadrakom, 1953) but in later studies, with a slightly different preparation of strips, it caused relaxation. Indeed, with just vascular smooth muscle, acetylcholine brings about contraction, in the same way as it does for airway smooth muscle. It binds M3 receptors, coupled to Gαq, and produces IP_3. When repeating the experiment with a sandwich of smooth muscle and endothelial cells, the results were the opposite. Robert Furchgott explained the paradox by the production of an endothelium-derived relaxing factor that overruled the direct effect of M3 on smooth muscle (Furchgott and Zawadzki, 1980). In his early preparations of arterial strips, the thin layer of endothelial cells had been accidentally removed (by rubbing or other mechanical force). The question remained, for about seven years, as to the identity of this relaxation factor. Two findings played a crucial role in elucidating its composition: one, that relaxation paralleled the production of cGMP (Rapoport and Murad, 1983) and two, that the enzyme that produces cGMP, guanylyl cyclase, could be activated by nitric oxide (Murad et al., 1978).

Nitric oxide is an endothelial relaxing factor

The endothelial relaxing factor is a gas, nitric oxide, produced by endothelial cells in response to a rise in Ca^{2+} which, through binding to calmodulin, activates NO-synthase (Ignarro et al., 1987; Palmer et al., 1987). Nitric oxide plays an essential role in intact organisms because blood pressure rises by 30% if you inhibit its synthesis or knock the gene out in mice (Shesely et al., 1996; Landesser and Drexler, 2007). Three genes code for nitric oxide synthase (NOS1–3), all of which are expressed in the human cardiovascular system. NOS1 (nNOS) is predominantly expressed in nerves. It is expressed in the autonomic nervous system and nerves that liberate nitric oxide qualify as nonadrenergic noncholinergic (NANC) nerves. NOS2 (or iNOS) is the inducible form expressed in numerous tissues and NOS3 (or eNOS) is prominently expressed in vascular endothelial cells (Qian and Fulton, 2013). It is worth noting that the *e*, *i*, and *s* nomenclature only has historical significance; we now know that all NO-synthases are widely expressed and may either be constitutive or inducible. In resting vascular endothelial cells, NO-synthase-3 is targeted to the plasma membrane by reversible palmitoylation on residues cys-15 and cys-26 (Shaul et al., 1996). Upon agonist stimulation, it is rapidly depalmitoylated and translocates from the plasma membrane to internal membrane structures.

NO-synthase is composed of two domains; a C-terminal reductase domain, which binds the prosthetic groups NADH, FMN, and FAD, and an N-terminal oxidase domain, which binds heme, zinc, tetrahydrobiopterin (BH4), and calmodulin. Endothelial NO-synthase forms a homodimer and this is essential for enzymatic activity. Electrons flow from the C-terminal reductase domain of one monomer to the N-terminal oxygenase domain of the other (Figure 4-22). The primary mode of activation is the binding of Ca^{2+}/Calmodulin, positioned between the reductase and oxidase domain. Ca^{2+} brings about a change in conformation that enables the flow of electrons from NADPH through FMN, FAD to BH4 and the heme in the oxygenase domain of the adjacent eNOS monomer. The arrival of an electron causes the reduction of oxygen and this starts a multistep process that ends up in the conversion of L-arginine into L-citrulline, water, and nitric oxide.

Nitric oxide stimulates soluble guanylyl cyclase

Nitric oxide is able to cross the plasma membrane and enters the surrounding tissue. It has a very short half-life, ranging from 10 to 30 s, during which it can diffuse a distance of 200–600 μm, enough to penetrate

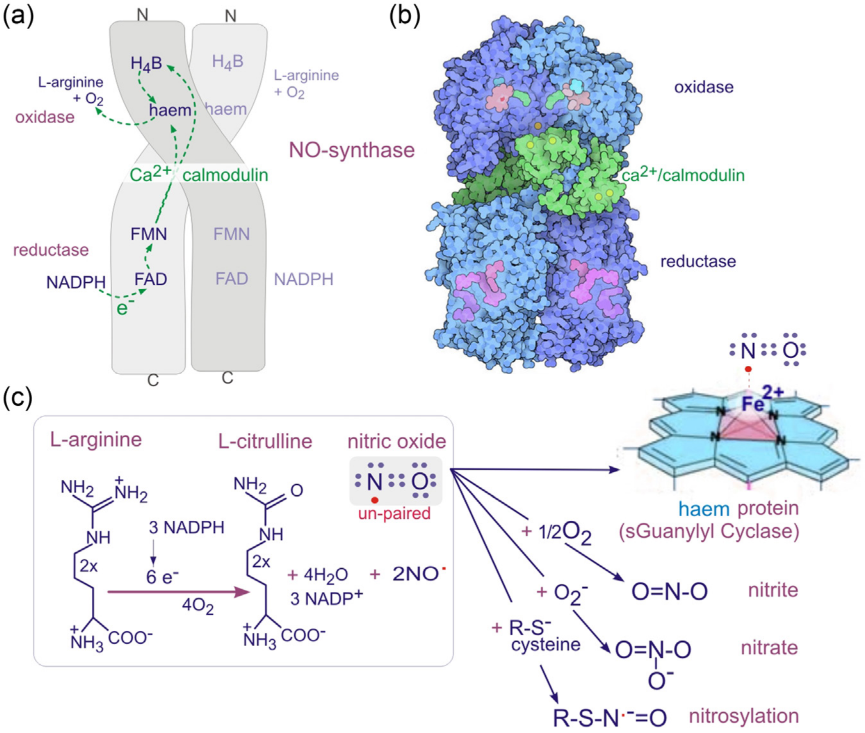


FIGURE 4-22 Structure–function relationship of NO-synthase. (a) NO-synthase occurs as a dimer. The reductase domain takes electrons from NADPH and shuttles them via prosthetic groups to the oxidase domain of the neighboring protein, where the electrons move onto oxygen. (b) This realistic representation is a compilation of three known structures, the reductase and oxidase domain and Ca^{2+}/calmodulin. Notice the strategic position of Ca^{2+}/calmodulin in the regulation of the transport of electrons (image from David Goodsell, published in "structural view of biology" (PDB-101) with permission from the Protein Data Bank (RCSB). (c) Production of nitric oxide from L-arginine and oxygen. Nitric oxide has one unpaired electron and this makes it a reactive molecule with a short half-life (in the order of tens of seconds). It can bind the ferrous group in heme (sharing one of its unpaired electrons), it forms nitrite with oxygen or nitrate with a superoxide anion or it interacts with a thiolate group of cysteine (R-S⁻) to form a nitrous thiol (process of nitrosylation).

multiple layers of smooth muscle cells. The passage in the blood is very likely to be much shorter because oxyhemo- and oxymyoglobin react with nitric oxide and convert it into nitrate (NO_3^-). It also can bind to hemo and myoglobulin to produce Hb-NO or Mb-NO.

In smooth muscle cells, the target is soluble guanylyl cyclase, an enzyme that converts GTP into cyclic GMP and PPi. Soluble guanylyl cyclase belongs to a small family of enzymes also including two retinal guanylyl cyclases (GUCY2D, GUCY2F) and two natriuretic peptide receptors (NPR1 and NPR2) (Figure 4-23). The soluble enzyme forms an asymmetric dimer composed of GUCY1A and GUCY1B. Their shared catalytic domain resembles the one of adenylyl cyclase, which is composed of C1- and C2-domains within a single protein (return to Chapter 3, Figure 3-11). With respect to the position of the catalytic residues, adenylyl- and guanylyl-enzymes precisely align with each other (Allerston et al., 2013). An important difference occurs, however, in the coordination of the bases of the nucleotide and this determines substrate specificity. Apart from the extensive contacts between the catalytic domain, the dimer is also held together by a PAS and a coiled-coil (CC) domain. Both the α- and the β-subunits contain a heme-binding domain but only one of the β-subunit (named βH-NOX) is functional. Nitric oxide binds to the proximal face of heme, thereby, replacing the connection with one axial ligand (a histidine) of Fe^{2+} (Figure 4-22(c)). This may flatten the heme but just how such changes are further transmitted to downstream catalytic events is still a major focus for study (Fritz et al., 2013; Friebe and Koesling, 2003; Nioche et al., 2004; Pellicena et al., 2004). Cytosolic concentration of cGMP rises to μM levels.

Membrane-bound guanylyl cyclases

Apart from the soluble guanylyl cyclase discussed above, the four other members of the family are membrane-bound enzymes. Their domain architecture reveals identical domains, an extracellular receptor segment and an intracellular segment containing a pseudokinase (not active), and a guanylyl cyclase domain (Figure 4-23(b)). In the case of the retinal guanylyl cyclase, the receptor does not bind ligand. Both types of transmembrane guanylyl cyclases must form a dimer in order to be active (one catalytic subunit does not suffice). The ANP receptors (NPR1 or -2) are held together by their extracellular domain and ligand binding is thought to cause a rotation of the intracellular domains which in turn activates the enzyme. The retinal guanylyl cyclase is activated by intracellular Ca^{2+}. The extracellular receptor segment may only serve to dimerize two complementary proteins. An additional PAS domain may reinforce this interaction.

The cGMP pathway leading to smooth muscle relaxation

The sequence of events that follows resemble what we have described for β2-agonists in the lung. Indeed, in the context of muscle contraction, nitric oxide can be best viewed as a functional antagonist of acetylcholine (Figure 4-24). Nitric oxide also functionally antagonizes noradrenaline acting on α1-receptors (ADRA1A) of smooth muscle cells in visceral arteries and arterioles (return to Chapter 3, Figure 3-28).

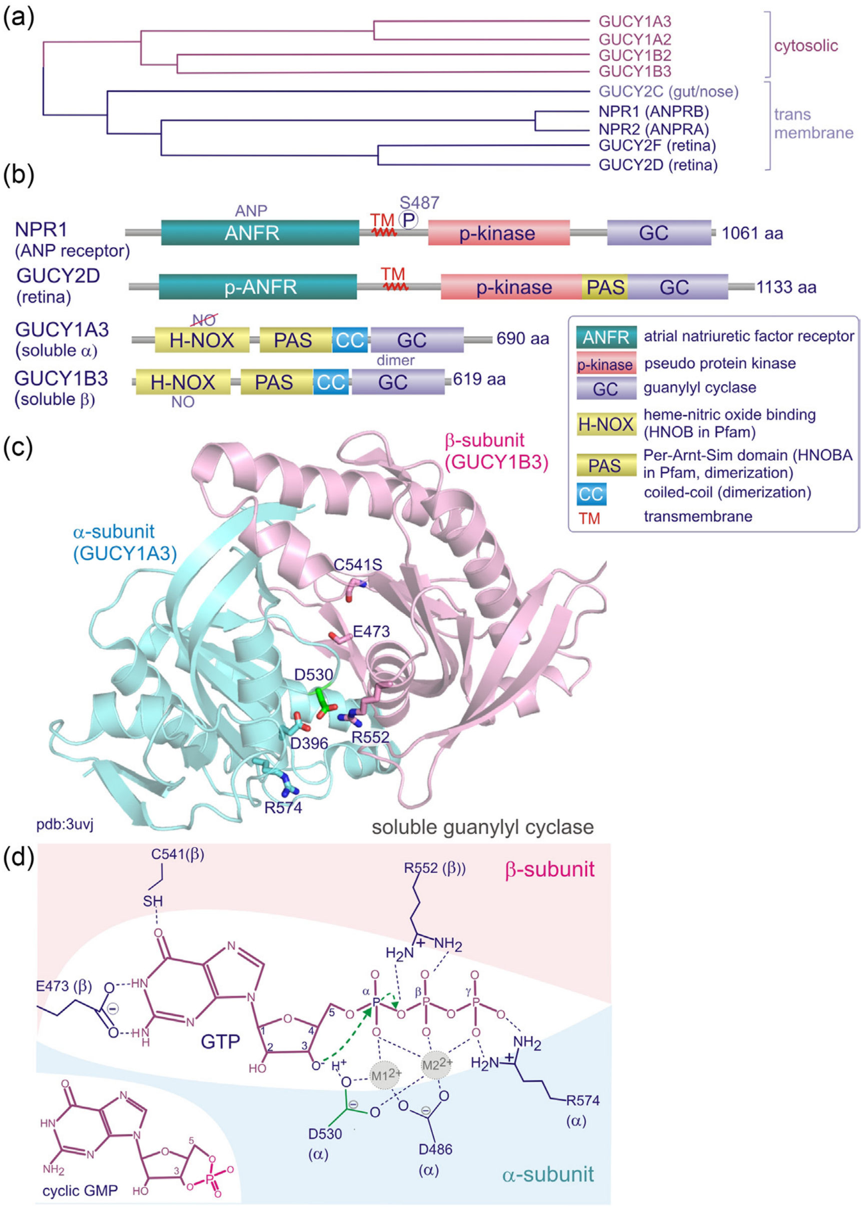


FIGURE 4-23 Members of the guanylyl cyclase and their mode of action. (a) The phylogenetic tree of human guanylyl cyclases shows the separation between cytosolic and transmembrane guanylyl cyclases. The transmembrane version is split into a genuine receptor and a retinal version with a pseudoreceptor domain. (b) The domain architecture of guanylyl cyclases illustrates the three groups, atrial natriuretic peptide (ANP) receptors, retinal guanylyl and soluble guanylyl cyclases. The receptor domain of the retinal cyclases is not functional (pseudo-domain) and the protein has acquired a PAS domain which may serve

to reinforce the interaction with a complementary protein (functional dimer). (c) Structure of the catalytic domain of the heterodimeric human soluble guanylyl cyclase 1, composed of two proteins, GYC1A3 (α-subunit) and GUCY1B3 (β-subunit). The positions of conserved amino acids are nearly equivalent to the amino acids in adenylyl cyclase. Substrate specificity is thought to occur through preferential binding of the guanine base. Pdb; 3uvj. (d) The active state of guanylyl cyclase, bound to a GTP analog, is not yet elucidated. The schematic representation of the coordination of GTP is based on positions of equivalent residues found in adenylyl cyclase. D486 and D530 coordinate the metal ions (shown as gray spheres) which serve to render the 3-hydroxy of the ribose more nucleophilic (by chasing away the H^+). Two arginines, R552 and R574, play a role in negative-charge compensation during the reaction. The ribose 3-O^- initiates the nucleophilic attack on α-phosphate (green-dotted arrow), leading to the generation of cAMP and PPi. The coordination of the purine ring by C541 and E473 is purely hypothetical.

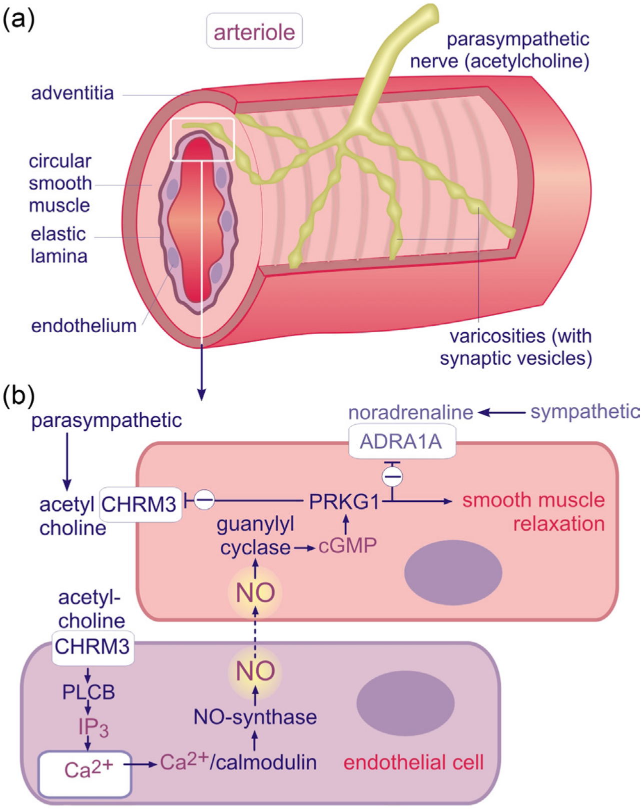


FIGURE 4-24 (a) Anatomy of an arteriole innervated by a parasympathetic nerve. Note that there are no specific junctions, as in skeletal muscle, neurotransmitter is freely released from varicosities. (b) Acetylcholine-mediated smooth muscle relaxation occurs through the intervention of nitric oxide produced by endothelial cells that line the vasculature. This is an example of functional antagonism, where the nitric oxide counteracts the vasoconstrictive action of both acetylcholine and noradrenaline (which signals through the α1-adrenoceptors (ADRA1A) in visceral arteries and arterioles).

Cyclic GMP is a second messenger, like cAMP, that interacts with proteins that carry a cyclic nucleotide-binding pocket. As targets we recognize a protein kinase, a number of PDEs and ion channels of the HCN family (return to Chapter 3, Figure 3-14). We start with the cGMP-dependent protein kinase (PRKG or PKG). It is widely expressed and exists in two variants PRKG1 and -2, each expressing splice variants (α, β, etc., versions). PRKG1 is most commonly involved in NO-mediated signaling (Francis et al., 2010). The two PRKG proteins share the same domain architecture, knowingly: an N-terminal-docking/dimerization domain (leucine-zipper), followed by a short autoinhibitory sequence (inhibitory substrate), two regulatory cyclic nucleotide-binding domains (CNBD-A and CNBD-B), a helical domain and finally a typical "AGC-kinase" catalytic domain (Figure 4-25). The catalytic domains of the cAMP- and cGMP-dependent protein kinases share 115 identical positions out of the 230 amino acids that constitute the domain, in other words they look alike (Kim et al., 2011; Osborne et al., 2011). The PRKG1 gene seems a fusion product of two genes, one coding for the catalytic- (PRKAC) and the other for the regulatory subunit (PRKAR1) of the cAMP-dependent protein kinase (return to Chapter 2, Figure 2-43 for the structure of PRKAC). Naturally, it may also be the other way around, that the PRKG1 was the precursor for the cAMP-dependent kinase holoenzyme. Cyclic GMP-dependent protein kinases are also sensitive to cAMP, albeit that they require 50- to 200-fold higher concentrations for full activation. Finally, binding of cGMP to the regulatory domains is thought to cause activation by removing the inhibitory substrate from the catalytic cleft followed by important changes in intermolecular contacts in the substrate-binding region (read, conformational changes) (Alverda et al., 2008). Structural data are still lacking.

PRKG1 brings about muscle relaxation by reducing the frequency of the Ca^{2+} oscillations and by making the contractile machinery less sensitive to Ca^{2+}. PRKG1 phosphorylates the inositol-1,4,5-trisphosphate receptor-associated cGMP kinase substrate (MRVI1, also abbreviated as IRAG) on residues ser-657 and ser-670 (Fritsch et al., 2004; Antl et al., 2007). It is a single-pass membrane protein which forms a trimeric complex with the IP_3-receptor (ITPR1) and PRKG1 (Figure 4-26; Schlossmann et al., 2000). Phosphorylation of MRVI1 attenuates the IP_3-evoked Ca^{2+} release from the sarcoplasmic reticulum. The importance of this protein has been made apparent by the observation that its deletion in mice prevents nitric oxide-mediated relaxation of smooth muscle (Desch et al., 2010). PRKG1 also phosphorylates the IP_3-receptor (ITPR1), on ser-1755, and this too reduces Ca^{2+}-release. It is not known how these events control gating of the Ca^{2+} pore. A third important substrate is the K^+ large-conductance channel KCNMA1, its phosphorylation causes increased conductance.

MRVI1 stands for murine retrovirus integration site-1 protein, a name so given because murine leukemia viral genomes (MLVs) tend to insert into the host genome at the 5′-end of this gene (among numerous other possible insertion sites). Viral integration causes a reduction of expression of MRVI1 but of other proteins as well and this may lead to murine myeloid leukemia. It was anticipated that MRVI1 could function as a tumor suppressor (which it is not). The MRVI1 protein is homologous to a lymphocyte-restricted membrane protein (LRMP or Jaw1) (Shaughnessy et al., 1999).

PRKG1 blocks the acetylcholine M3-signaling pathway by phosphorylation of RGS2, a regulator of G-protein signaling. RGS2 is a protein kinase that phosphorylates G-protein-coupled receptors and arrests their signaling through subsequent binding of one of the arrestins. Phosphorylation of RGS2 enhances its activity and this, in turn, enhances its capacity to phosphorylate the M3 receptor and block its vasoconstrictive action (Ruth et al., 1993). This event is not universal as it was not observed in bovine tracheal smooth muscle.

PRKG1 also reduces sensitivity for Ca^{2+} by enhancing the activity of myosin light-chain phosphatase. It phosphorylates the regulatory subunit PPP1R12A on ser-695 and this was shown to prevent the inhibitory phosphorylation of ROCK1 and PRKC (on thr-696) (Wooldridge et al., 2004). PRKG1 forms a complex with the phosphatase subunit; the dimerization domain (DD) of PRKG1 either binds the C-terminal region (a leucine zipper (1004–1028 aa) or the N1N2-coiled coil domain (850–1004) (Figure 4-26).

The last substrate we deal with is PDE5A, whose phosphorylation at ser-99, enhances affinity for binding of cGMP to the allosteric regulation domain (GAF-A). This leads to stimulation and a subsequent loss of cGMP.

Web resource

KEGG, Kyoto Encyclopedia of Genes and Genomes

- Vascular smooth muscle contraction map04270 (http://www.genome.jp/kegg-bin/show_pathway?map04270)

Nitrosylation of proteins by nitric oxide

High levels, in the order of μM, of nitric oxide can cause nitrosylation of cysteines, where -SH is replaced by -SNO (a nitrous thiol, indicated by SNO) (Figure 4-22). Proteins near the site of production of nitric oxide are

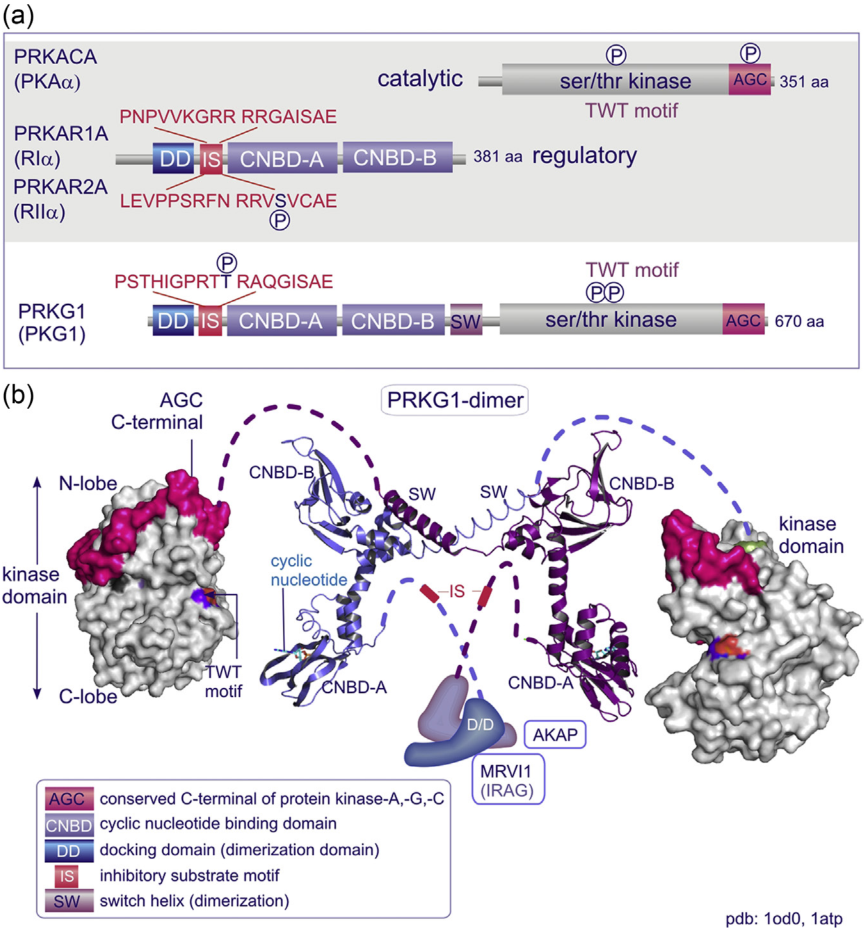


FIGURE 4-25 Domain architecture of cyclic nucleotide-dependent protein kinases. (a) The cyclic AMP-dependent protein kinases holoenzyme comprises a catalytic (PRKAC) and a regulatory subunit (PRKAR). Each exist in different isoforms (PRKACA, -B, -G, and PRKAR1A, -2A respectively). The regulatory subunit carries a dimerization domain (DD), an inhibitory substrate motif (IS), and two cyclic nucleotide-binding domains (CNBD). The catalytic domain contains a TWT motif positioned in the activation segment and which need to be phosphorylated in order to achieve full activity. It also carries a characteristic AGC-kinase C-terminal. Binding of the cyclic nucleotide causes the separation of the two proteins. For PRKG, the catalytic and regulatory subunits are in one and the same protein. Two isoforms exist, PRKG1 and -2. The first plays a role in smooth muscle relaxation. (b) Partial molecular structure of PRKG1. The protein kinase functions as a dimer, held together by the dimerization domain (DD) and the switch helix (SW). Regulation of the catalytic domain occurs in the same fashion as for PKRAC. Cyclic-GMP separates the kinase domain from the inhibitory substrate motif (IS). The representation shown below is hypothetical because the structure of the intact protein kinase (active or inactive) is not known. The kinase domain is a surface representation of PRKAC. The dimerization domain interacts with MRVI1 and in analogy with cAMP-dependent protein kinase, may interact with AKAPs. Pdb: 3od0, 1atp.

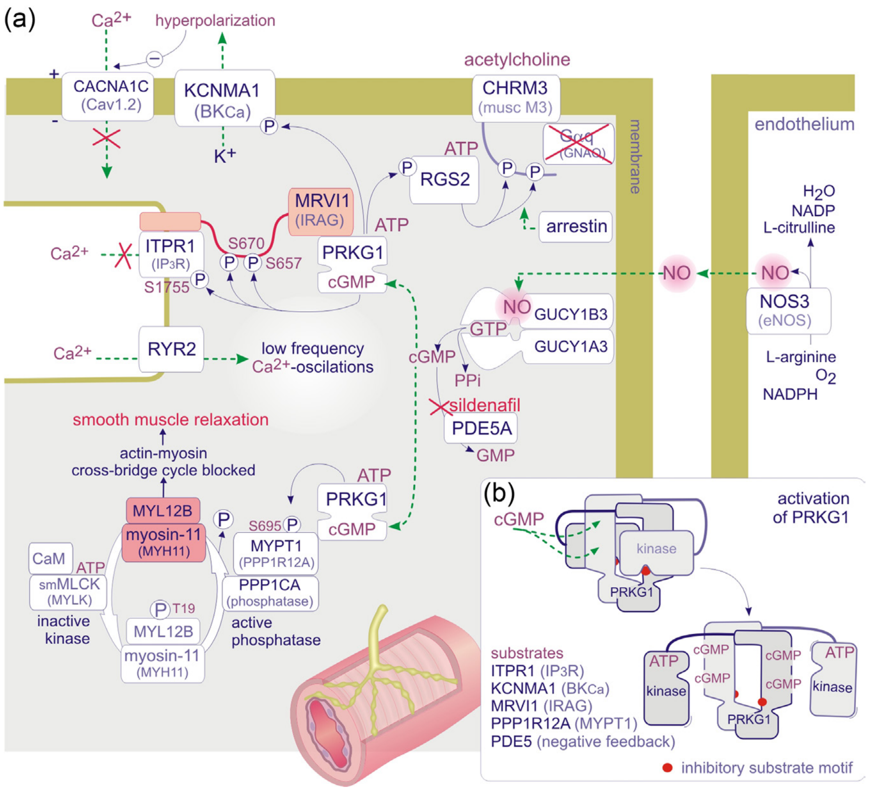


FIGURE 4-26 The nitric oxide pathway to vascular smooth muscle relaxation. (a) Nitric oxide, produced by endothelial cells, diffuses into the smooth muscle cell and binds the heme-prosthetic group on guanylyl cyclase (dimer of GUCY1A3 and -B3). This causes the production of the second messenger cyclic-GMP. It diffuses into the cell and binds the regulatory domain of the cGMP-dependent protein kinase (PRKG1) and causes its activation. The dimerization domain of this protein kinase attaches it to two of its substrates. The first is MRVI1, a transmembrane protein associated with the inositol-3,4,5-phosphate receptors (ITPR1). PRKG1 phosphorylates MRVI1 on two residues (S657 and S670) and it phosphorylates IPTR1 on S1755. Both phosphorylations lead to a reduced conductance of Ca^{2+}, thus inhibiting the intracellular Ca^{2+} oscillations. As a consequence, the Ca^{2+}/calmodulin-dependent myosin light-chain kinase (MYLK) is greatly reduced. RPKG1 also phosphorylates KCNMA1, a Ca^{2+}-sensitive K^+ large-conductance channel, and increases its permeability. The ensuing hyperpolarization blocks the inward Ca^{2+}-current through CACNA1C. This too prevents the development of high frequency Ca^{2+} oscillations. PRKG1 phosphorylates RGS2, a protein kinase that phosphorylates the intracellular loop of the acetylcholine M3 receptor and thereby prevents interaction with G-proteins. Finally, PRKG1 phosphorylates the regulatory subunit of myosin light-chain phosphatase (PPP1R12A) on S695 and this prevents inhibitory phosphorylations by ROCK1 or PRKC. Low kinase- and high phosphatase-activity cause dephosphorylation of the regulatory myosin light-chain (MYL12B) with the result of blocking the actin–myosin cross-bridge cycle. The smooth muscle gradually relaxes. (b) Schematic representation of a possible mode of activation of PRKG1. In the absence of cGMP, the kinase domain binds the regulatory segment and becomes inhibited by the inhibitory substrate motif (as is the case for PRKACA). Binding of cGMP dislocates the kinase domain. Further conformational changes are anticipated in order to bring the kinase in a fully active state.

certainly affected. The reaction is reversible but requires two enzymes, S-nitrosoglutathione reductase and thioredoxin 1 (TXN) (Qian and Fulton, 2013). In vascular smooth muscle cells, NO-synthase is nitrosylated on cys-101 and it reduces enzyme activity, thus serving as a negative feedback mechanism (Erwin et al., 2005). Many other proteins can be nitrosylated and the process affects numerous cellular events which we will not elaborate (subject reviewed in Qian and Fulton (2013) and Lima et al. (2010)).

Robert Furchgott, Louis Ignarro, and Ferid Murad were awarded the 1998 Nobel Prize in Physiology or Medicine "for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system."

NO synthase also plays a role in inhibition of platelet aggregation, leukocyte adhesion, and endothelial cell proliferation and it is involved in the killing of microbes by macrophages.

A step-by-step guide to nitric oxide-mediated penile erection

PDE inhibitors became a therapeutic target about 1987, soon after their vasodilatory action was reported in the literature (Schoeffter et al., 1987). Staff at Pfizer sought to apply them for treatment of angina. In the clinical trial with sildenafil, considered a selective PDE5 inhibitor, participants reported important positive effects on penile erection. Once Pfizer recognized sildenafil's "adverse effect," they started considering it for treatment of male impotence and the new trial, with quite a different experimental setup and endpoint, provided satisfactory results. It was marketed as Viagra in 1998 (Boolell et al., 1996).

Penile erection results from elevated pressure in the corpora cavernosa, two spongy sinusoidal tissues that run the length of the penis. They contain numerous trabeculae, smooth muscle cell structures that traverse the cavity, and are surrounded by the tunica albuginea, an elastic membrane. Blood supply occurs through the arteria penis profunda, a branch of the arteria pudenda interna. Blood is evacuated via a venous plexus, the vena circumflexa, situated outside the tunica albuginea and underneath the fascia profundis. These veins drain into the vena dorsalis penis profunda. The arteries and arterioles as well as the trabeculae are innervated both by sympathetic and parasympathetic nerves. The sympathetic nerve originates from L1 and L2 (lumbar spine), whereas the parasympathetic originates from S2–4 (sacral spine), collectively named the nervi erigentes or pelvic nerves. One branch of the pelvic nerves, the nervus pudendus, follows the arteria pudenda interna. It becomes the nervus dorsalis penis, which innervates the skin and the glans penis, and one of its branches enters the corpus cavernosum. The sympathetic neurons release noradrenaline and cause contraction through the α1-receptor (return to Chapter 3, Figure 3-28). The parasympathetic nerves carry a mixture of neurotransmitters: vasoactive-intestinal polypeptide, acetylcholine and they produce nitric oxide (Hedlund et al., 1999). The release of nitric oxide, either from endothelial cells or directly from the neurons, greatly augments the blood supply, consequence of dilatation of the vasculature, and it causes the relaxation of the trabeculae in the corpus cavernosum. Sufficient swelling compresses the emissary veins (vena circumflexa) thereby blocking outflow. In the case of erectile dysfunction, this blockage is not attained and the penis does not erect (Figure 4-27). Blocking PDE5A, which is highly expressed in the pelvic area

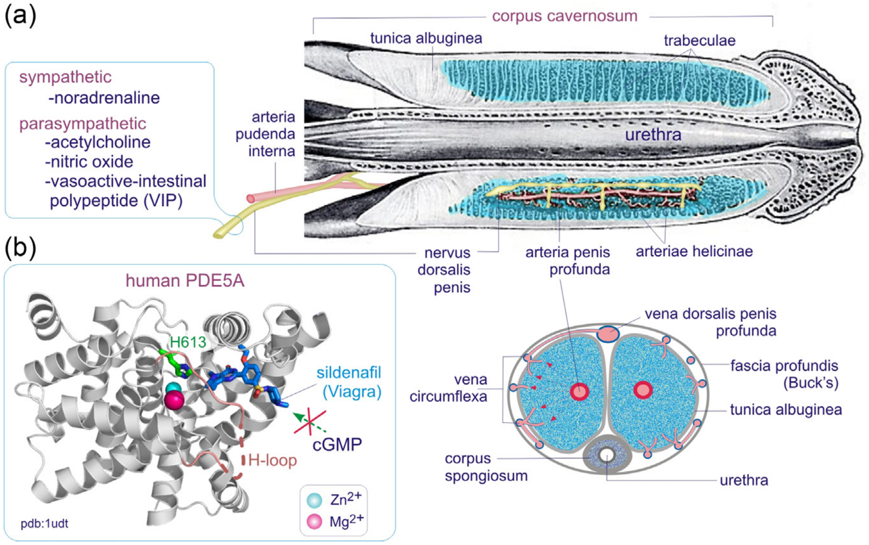


FIGURE 4-27 Regulation of smooth muscle contraction in the corpus cavernosum of the penis. The corpus cavernosum is a spongy tissue surrounded by the tunica albuginea. It contains numerous trabeculae, smooth muscle cell structures that traverse the cavity. The corpus cavernosum is innervated by both sympathetic and parasympathetic nerves that are carried by the nervus pudendus, of which one of its branches, the nervus dorsalis penis, enters the spongy tissue. Blood supply occurs through the arteria penis profunda, a branch from the arteria pudenda interna. The sympathetic nerves produce noradrenaline, which through α1-receptors on smooth muscle causes vasoconstriction. The parasympathetic nerves produce a mixture of neurotransmitters. Both acetylcholine, through the production of nitric oxide by vascular endothelial cells, and nitric oxide produced by the nerves, cause smooth muscle cell relaxation by stimulating the production of cyclic-GMP. Penile erection occurs through swelling of the corpus cavernosum, a process in which the compression of emissary veins (vena circumflexa) plays an essential role. Impotence is the consequence of an insufficient relaxation of smooth muscle, in particular the trabeculae, hence preventing a sufficient compression of the emissary veins. (b) Sildenafil, marketed as Viagra, occupies the catalytic site (indicated by His-613) of phosphodiesterase-5A and prevents the conversion of cGMP into GMP. Inhibition of PDE5A boosts the vasodilatory action of the cGMP-dependent protein kinase PRKG1 and facilitates penile erection. *Image of the longitudinal section of the penis adapted from Sobotta and Becher (1975).*

and in the lungs, boosts the nitric oxide response (Figure 2-26). Sildenafil does inhibit retinal PDE6 to some extent and causes blue tinting of vision.

Presently, three PDE5 inhibitors are on the market: sildenafil (Viagra, Pfizer), vardenafil (Levitra, Bayer-GSK), and tadalafil (Cialis, IC351, Lilly-ICOS). The inhibitors are tested for different therapeutic purposes. Sildenafil was shown to induce neurogenesis and promote functional recovery after stroke in rat, to be effective in hypoxia-induced pulmonary hypertension in rat, and to improve endothelium-dependent vasodilatation in smokers. Furthermore, sildenafil and vardenafil enhance early memory consolidation of object information and, lastly, vardenafil and tadalafil are able to induce caspase-dependent apoptosis in B-chronic lymphocytic leukemia cells and are considered for chemotherapy.

PILLS FOR THE BOYS?

The explosive sales of Viagra are not justified by its sole use by impotent men. Indeed, further studies showed that Viagra also had a positive effect on sexual desire. Apparently, for men, pelvic blood flow, desire, and “performance” are inseparable. Incidentally, a similar relationship has been revealed in studies with cynomolgus monkeys. In this experimental setting, erectile function was not improved by providing Viagra but by inserting the large-conductance K^+ channel (KCNMA1) into the smooth muscle cells of the corpus cavernosum (Christ et al., 2009). Sexual satisfaction is not just a man’s affair and Viagra has been tested with women, in laboratory experimental settings, but the findings are much less convincing. The nervous innervation and vascular supply of the vagina are more or less similar to men and, indeed, Viagra was found to be effective in enhancing vaginal engorgement (clitoral erection) during erotic stimulation. However, pelvic flow was not conclusively associated with sexual desire, sensation, lubrication, or satisfaction (Basson et al., 2002; Schoen and Bachman, 2009; Laan et al., 2002). The search continues!

GLYCERYL NITRATE, NITRIC OXIDE, AND ANGINA PECTORIS

The ability of organic nitrates to relieve the pain of angina pectoris dates back to 1867 when the English physician Lauder Brunton treated his patients with vapors of amyl nitrate and William Murrell experimented with glyceryl trinitrate tablets. Angina pectoris (from Latin, *angere* signifying tighten or strangling and *pectis* meaning chest) occurs when the oxygen supply to the heart muscle is insufficient to its needs. The resulting

continued

GLYCERYL NITRATE, NITRIC OXIDE, AND ANGINA PECTORIS *(cont'd)*

pain is localized in the chest, left arm, and neck. Three types of angina are recognized: (1) stable angina, with predictable pain during exercise and being the result of narrowing of the coronary artery due to the formation of atheroma (atherosclerosis); (2) unstable angina, where pain occurs during moderate exercise or at rest, and is the result of ruptured atheroma which gives rise to blood clots that occlude the coronary artery; and (3) variable angina, a rare phenomenon due to spasm of the coronary artery. Nitrates cause relief because, after reduction, they produce nitric oxide and cause vasodilatation.

Currently, in France, patients entering the emergency ward with angina symptoms, immediately receive glyceryl trinitrate tablets, or the equivalent isosorbide mononitrate, which are placed under the tongue (sublingual administration). Their life-saving action is explained by two mechanisms: vasodilation of collateral arteries of the coronary artery, causing improvement of coronary perfusion, and a decreased left ventricular load which diminishes the energy/oxygen demand of the myocardium. Molsidomine, another nitric oxide donor, is orally administered to patients with stable angina in order to improve cardiac performance (while waiting for surgery). It is also (and especially) used in patients with severe cardiac insufficiency (a condition where blood accumulates in the lungs and causes edema) in order to reduce breathlessness. The development of tolerance is the main drawback of these treatments. Finally, aspirin (an inhibitor of phospholipase A2) is routinely prescribed in patients with instable angina because it reduces the risk of blood clotting.

Glyceryl trinitrate is the same as nitroglycerine, nothing less than the explosive ingredient of dynamite. Alfred Nobel became famous for his discovery that "kieselguhr" sand, produced by the skeletons of diatoms (siliceous algae), stabilized nitroglycerine and allowed it to be handled more safely. This invention earned him the fortune with which he established, in 1895, the eponymous prize (Nobel Prize). As an anecdote, the factory workers of Nobel suffered from headaches, which we now know is an adverse effect of vasodilatation. Advancing in the week, the pain diminished, due to tolerance, and reappeared on Monday morning after a weekend without exposure. Alfred Nobel himself suffered from angina pectoris but he was reluctant to follow the recommendations of Brunton and Murrell, for he knew all too well that it caused headaches (doses are more moderate these days). Instead of dying from a heart attack, he suffered a cerebrovascular accident and died in 1896 at the age of 63.

NEUROTRANSMITTERS THAT FUNCTION WITH BOTH IONOTROPIC AND METABOTROPIC SIGNALING MECHANISMS

Acetylcholine is not the only ligand for which the binding pocket has been inserted in two types of receptors: a ligand-gated ion channel (ionotropic) and a seven-transmembrane G-protein-coupled receptor (metabotropic). Other examples are ATP, GABA, glutamate, and 5-hydroxytryptamine (serotonin). They too bind two entirely different receptor types (Figure 4-28).

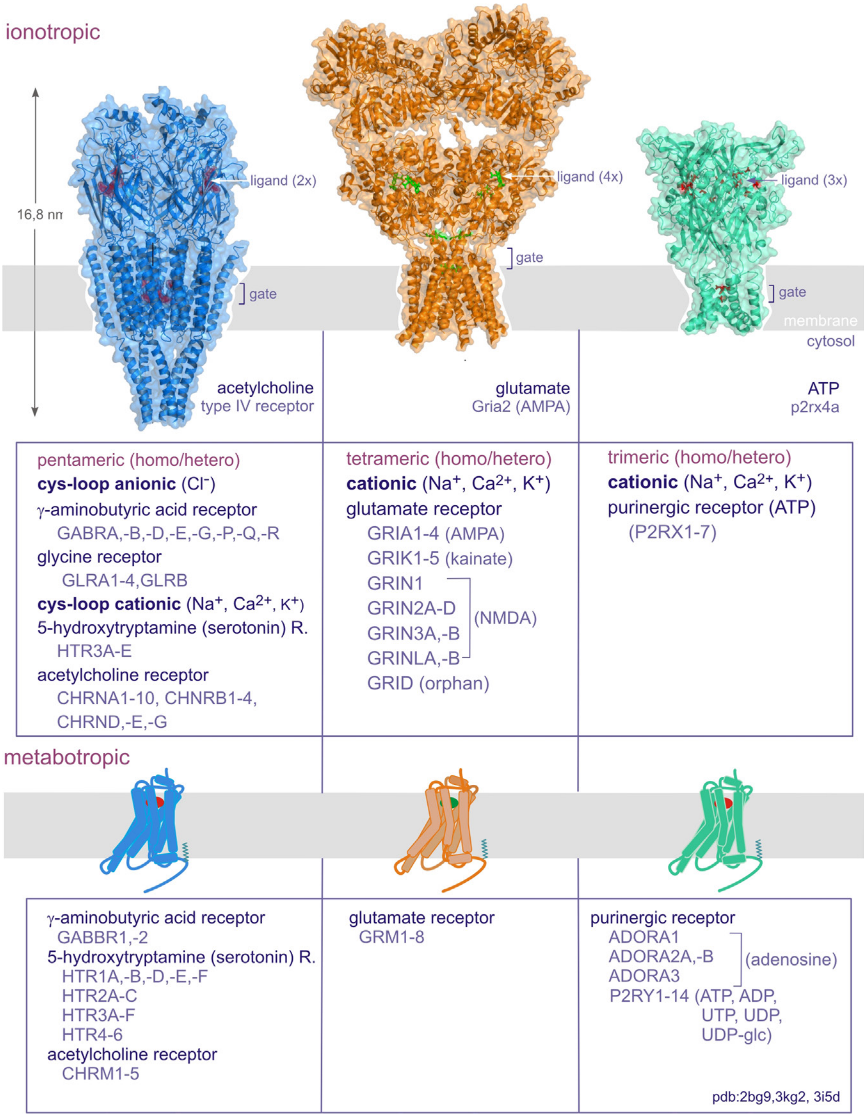


FIGURE 4-28 Neurotransmitters acting on both iono- and metabotropic receptors. Glycine is an exception in the list, it lacks a metabotropic counterpart.

The ionotropic receptors play essential roles in the regulation of frequency of action potentials in the nervous system. Some of the channels are permeable to Ca^{2+} and they directly contribute to the regulation of gene transcription necessary for learning and memory. Ionotropic receptors also occur in nonexcitable cells. The ionotropic receptors constitute three different receptor types: the pentameric "cys-loop" receptors, which have the same structure and gating mechanisms as described here for the nicotinic acetylcholine receptor, the tetrameric glutamate receptor, and the trimeric ATP receptor. The glycine receptor is included in the list of ionotropic receptors but it lacks a metabotropic counterpart (Nys et al., 2013). The metabotropic receptors signal through heterotrimeric G-proteins followed by the production of second messengers. They involve the pathways initiated by adenylyl cyclase or phospholipase C and the many arrestin-mediated signaling events.

References

Albuquerque, E.X., Pereira, E.F., Alkondon, M., Rogers, S.W., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89, 73–120.

Allerston, C.K., von Delft, F., Gileadi, O., 2013. Crystal structure of the catalytic domain of the heterodimeric human soluble guanylate cyclase. Plos One 8, e5764.

Alverda, V., Mazon, H., Versluis, C., Hemrika, W., Esposito, G., van de Heuvel, R., Scholten, A., Heck, A.J.R., 2008. cGMP-binding prepares PKG for substrate binding by disclosing the C-terminal domain. J. Mol. Biol. 375, 1380–1393.

Antl, M., von Bruhl, M.L., Eiglsperger, C., Werner, M., Konrad, I., Kocher, T., Wilm, M., Hofmann, F., Massberg, S., Schlossmann, J., 2007. IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. Blood 109, 552–559.

Ashley, C.C., Ridgway, E.B., 1968. Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibers. Nature 219, 1168–1169.

Bai, Y., Sanderson, M.J., 2006. Modulation of the Ca^{2+} sensitivity of airway smooth muscle cells in murine lung slices. Am. J. Physiol. Lung Cell Mol. Physiol. 291, L208–L221.

Basson, R., McInnes, R., Smith, M.D., Hodgson, G., Koppiker, N., 2002. Efficacy and safety of sildenafil citrate in women with sexual dysfunction associated with female sexual arousal. J. Womens Health Gend. Based Med. 11, 367–377.

Beavo, J.A., 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. 75, 725–748.

Beghe, B., Rabe, K.F., Fabbri, L.M., 2013. Phosphodiesterase-4 inhibitor therapy for lung diseases. Am. J. Respir. Crit. Med. 188, 271–278.

Bender, A.T., Beavo, J.A., 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol. Rev. 58, 488–520.

Bernard, C., 1956. Analyse physiologique des propriétés des systèmes musculaire et nerveux au moyen du curare. C. R. Hebd. Acad. Sci. 43, 825–829.

Binz, T., Sikorra, S., Mahrhold, S., 2010. Clostridial neurotoxins: mechanism of SNARE cleavage and outlook on potential substrate specificity reengineering. Toxins 2, 665–682.

Boolell, M., Gepi-Attee, S., Gingell, J.C., Allen, M.J., 1996. Sildenafil, a novel effective oral therapy for male erectile dysfunction. Br. J. Urol. 78, 257–261.

Brejc, K., van Dijk, W.J., Klaassen, R.V., Schurmanns, M., van der Oost, J., Smit, A.B., Sixma, T.K., 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411, 269–276.

Brisson, A., Unwin, P.N., 1984. Tubular crystals of acetylcholine receptor. J. Cell Biol. 99, 1202–1211.

Brook, R.D., Julius, S., 2000. Autonomic imbalance, hypertension, and cardiovascular risk. J. Hypertens. 13, 112S–122S.

Burden, S.J., Sargent, P.B., McMahan, U.J., 1979. Acetylcholine receptors in regenerating muscle accumulate at the original synaptic sites in the absence of the nerve. J. Cell Biol. 82, 412–425.

Butcher, R.W., Sutherland, E.W., 1962. Adenosine 3′, 5′phosphate in biological materials: 1. Purification and properties of cyclic 3′, 5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′, 5′-phosphate in human urine. J. Biol. Chem. 237, 1244–1250.

Cancedo, J.M., 2013. Biological roles of cAMP, variations on a theme in the different kingdoms of life. Biol. Rev. 88, 645–668.

Celie, P.H.N., Van Rossum-Fikkert, S.E., van Dijk, W.J., Brejc, K., Smith, A.B., Sixma, T.K., 2004. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. Neuron 41, 907–914.

Celie, P.H., Kasheverov, I.E., Mordvintsev, D.Y., Hogg, R.C., van Nierop, P., van Elk, R., van Rossum-Fikkert, S.E., Zhmak, M.N., Bertrand, D., Tsetlin, V., Sixma, T.K., Smit, A.B., 2005. Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an alpha-conotoxin PnIA variant. Nat. Struct. Mol. Biol. 12, 582–588.

Changeux, J.P., Kasai, M., Lee, C.Y., 1970. Use of a snake venom toxin to characterize the cholinergic receptor protein. PNAS U.S.A. 67, 1241–1247.

Christ, G.J., Andersson, K.E., Williams, K., Zhao, W., D'Agostino Jr., R., Kaplan, J., Aboushwareb, T., Yoo, J., Calenda, G., Davies, K.P., Sellers, R.S., Melman, A., 2009. Smooth-muscle-specific gene transfer with the human maxi-k channel improves erectile function and enhances sexual behavior in atherosclerotic cynomolgus monkeys. Eur. Urol. 56, 1055–1066.

Clapham, D.E., 2003. TRP channels as cellular sensors. Nature 426, 517–524.

Conn, P.J., Jones, C.K., Lindsley, C.W., 2009. Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol. Sci. 30, 148–155.

Connolly, C.N., Wafford, K.A., 2004. The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Biochem. Soc. Trans. 32, 529–534.

Coote, J.H., 2013. Myths and realities of the cardiac vagus. J. Physiol. 591, 4073–4085.

Dale, H.H., 1914. The action of certain esters and ethers of choline, and their relation to muscarine. J. Pharmacol. Exp. Ther. 6, 147–190.

Desch, M., Sigl, K., Hieke, B., Salb, K., Kees, F., Bernhard, D., Jochim, A., Spiessberger, B., Höcherl, K., Feil, R., Feil, S., Lukowski, R., Wegener, J.W., Hofmann, F., Schlossmann, J., 2010. IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation. Cardiovasc. Res. 86, 496–505.

Deshpande, D.A., Theriot, B.X., Penn, R.B., Walker, J.K., 2008. Beta-arrestins specifically constrain beta2-adrenergic receptor signalling and function in airway smooth muscle. FASEB J. 22, 2134–2141.

DiFranco, M., Quinonez, M., Vergara, J.L., 2012. The delayed rectifier potassium conductance in the sarcolemma and the transverse tubular system membranes of mammalian skeletal muscle fibers. J. Gen. Physiol. 140, 109–137.

Doyle, D.A., Moras-Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., MacKinnon, R., 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280, 69–77.

Dror, R.O., Green, H.F., Valant, C., Borhani, D.W., Valcourt, J.R., Pan, A.C., Arlow, D.H., Canals, M., Lane, J.R.L., Rahmani, R., Baell, J.B., Sexton, P.M., Christopoulos, A., Shaw, E.H., 2013. Structural basis for modulation of a G-protein-coupled receptor by allosteric drugs. Nature 503, 295–299.

Dunnill, M.S., 1960. The pathology of asthma, with special reference to changes in the bronchial mucosa. J. Clin. Pathol. 13, 27–33.

Einthoven, W., 1892. Über die Wirkung der Bronchial muskeln, nach einer neuen methode untersucht, und über Asthma nervosum. Pflügers Arch. 51, 367–444.

Ellerbroek, S.M., Wennerberg, K., Burridge, K., 2003. Serine phosphorylation negatively regulates rhoA in vivo. J. Biol. Chem. 278, 19023–19031.

Erneux, C., Govaerts, C., Communi, D., Pesesse, X., 1998. The diversity and possible functions of the inositol 5-polyphosphatases. Biochim. Biophys. Acta 1436, 185–199.

Erwin, P.A., Mithcell, D.A., Sartoretto, J., Marletta, M.A., Michel, T., 2005. subcellular targeting and differential S-nitrosylation of endothelial nitric-oxide synthase. J. Biol. Chem. 281, 151–157.

Fox, G.Q., Richardson, G.P., 2004. The developmental morphology of Torpedo marmorata: electric organ-myogenic phase. J. Comp. Neurol. 179, 677–697.

Francis, S.H., Busch, J.L., Corbin, J.D., 2010. cGMP-dependent protein kinase and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol. Rev. 62, 525–563.

Friebe, A., Koesling, D., 2003. Regulation of nitric oxide sensitive guanylyl cyclase. Circ. Res. 93, 96–105.

Fritsch, R.M., Saur, D., Kurjak, M., Oesterle, D., Schlossmann, J., Geiselhoringer, A., Homann, F., Allescher, H.D., 2004. InsP3R-associated cGMP kinase substrate (IRAG) is essential for nitric oxide-induced inhibition of calcium signalling in human colonic smooth muscle. J. Biol. Chem. 279, 12551–12559.

Fritz, B.G., Robers, S.A., Ahmed, A., Breci, L., Li, W., Weichsel, A., Brailey, J.L., Wysocki, V.H., Tama, F., Montfort, W.R., 2013. Molecular model of a soluble guanylyl cyclase fragment determined by small-angle X-ray scattering and chemical cross-linking. Biochem 52, 1568–1582.

Frizzell, R.A., Hanrahan, J.W., 2012. Physiology of epithelial chloride and fluid secretion. Cold Spring Harb. Perspect. Med. 2, a009563.

Furchgott, R.F., Bhadrakom, S., 1953. Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. J. Pharmacol. Exp. Ther. 108, 129–143.

Furchgott, R.F., Zawadzki, J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373–376.

Galli, S.J., Tsai, M., Piliponsky, A.M., 2008. The development of allergic inflammation. Nature 454, 445–454.

Haga, K., Haga, T., 1985. Purification of the muscarinic acetylcholine receptor from porcine brain. J. Biol. Chem. 260, 7927–7935.

Haga, K., Haga, T., Ichiyama, A., et al., 1985. Functional reconstitution of purified muscarinic receptors and inhibitory guanine nucleotide regulatory protein. Nature 316, 731–733.

Haga, K., Kruze, A.C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., Weis, W.I., Okada, T., Kobilka, B., Haga, T., Kobayashi, T., 2012. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. Nature 482, 547–551.

Hansen, S.B., Tao, X., MacKinnon, R., 2011. Structural basis of PIP2 activation of the classical inward rectifier K^+ channel Kir2.2. Nature 477, 495–498.

Hecht, S.S., 1999. Tobacco smoke carcinogens and lung cancer. J. Natl. Cancer Inst. 91, 1194–1210.

Heckman, C.J., Enoka, R.M., 2012. Motor Unit. Compr. Physiol. 2, 2629–2682.

Hedlund, P., Alm, P., Andersson, K.E., 1999. NO synthase in cholinergic nerves and NO-induced relaxation in the rat isolated corpus cavernosum. Br. J. Pharmacol. 127, 349–360.

Heidman, T., Changeux, J.P., 1984. Time-resolved photolabeling by the non-competitive blocker chlorpromazine of the acetylcholine receptor in its transiently open and closed ion channel conformations. PNAS U.S.A. 81, 1897–1901.

van Helden, H.P., Busker, B.P., Bruijnzeel, P.L., 1996. Pharmacological effects of oximes: how relevant are they? Arch. Toxicol. 70, 779–786.

Hernandez, C.M., Dineley, K.T., 2012. α7 nicotinic acetylcholine receptors in Alzheimer's disease: neuroprotective, neurotrophic or both? Curr. Drug Targets 13, 613–622.

Hung, R.J., McKay, J.D., Gaborieau, V., Boffetta, P., Hashibe, M., Zaridze, D., et al., 2008. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. Nature 452, 633–637.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G., 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. PNAS 84, 9265–9269.

Jensen, M.T., Suadicani, P., Hein, H.O., Gyntelberg, F., 2013. Elevated resting heart rate, physical fitness and all-cause mortality: a 16-year follow-up in the Copenhagen male study. Heart 99, 882–887.

Katz, B., Thesleff, S., 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. J. Physiol. 138, 63–80.

Khromov, A.S., Momotani, K., Jin, L., Artamonov, M.V., Shannon, J., Eto, M., Somlyo, A.V., 2012. Molecular mechanism of telokin-mediated disinhibition of myosin light chain phosphatase and cAMP/cGMP-induced relaxation of gastrointestinal smooth muscle. J. Biol. Chem. 287, 20975–20985.

Kim, J.J., Casteel, D.E., Huang, G., Kwon, T.H., Ren, R.K., Zwart, P., Headd, J.J., Brown, N.G., Chow, D.C., Palzkill, T., Kim, C., 2011. Co-crystal structures of PKG1β (92–227) with cGMP and cAMP reveal the molecular details of cyclic nucleotide binding. Plos One 6, e1841.

Koh, S.D., Sanders, K.M., Carl, A., 1996. Regulation of smooth muscle delayed rectifier K^+ channels by protein kinase A. Pflügers Arch. 432, 401–412.

Krishna, S., Zhong, X.-P., 2013. Regulation of lipid signaling by diacylglycerol kinases during T cell development and function. Front. Immunol. 4 article 178.

Kruse, A.C., Hu, J., Pan, A.C., Arlow, D.H., Rosenbaum, D.M., Rosemond, E., et al., 2012. Structure and dynamics of the M3 muscarinic acetylcholine receptor. Nature 482, 552–556.

Kubo, T., Fukuda, K., Mikami, A., et al., 1986. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. Nature 323, 411–416.

Laan, E., van Lunsen, R.H., Everaerd, W., Riley, A., Scott, E., Boolell, M., 2002. The enhancement of vaginal vasocongestion by sildenafil in healthy premenopausal women. J. Womens Health Gend. Based Med. 11, 357–365.

Landesser, U., Drexler, H., 2007. Endothelial function and hypertension. Curr. Opin. Cardiol. 22, 316–320.

Langley, J.N., 1905. On the reaction of nerve cells and nerve endings to certain poisons chiefly as regards the reaction of striated muscle to nicotine and to curare. J. Physiol. (London) 33, 374–473.

Langley, J.N., 1907. On the contraction of muscle, chiefly in relation to the presence of "receptive" substances. J. Physiol. 36, 347–384.

Lewis, M.J., Short, A.L., Lewis, K.E., 2006. Autonomic nervous system control of the cardiovascular and respiratory systems in asthma. Respir. Med. 100, 1688–1705.

Li, L., Eto, M., Lee, M.R., Morita, F., Yazawa, M., Kitazawa, T., 1998. Possible involvement of the novel CPI-17 protein in protein kinase C signal transduction of rabbit arterial smooth muscle. J. Physiol. 508, 871–881.

Li, Y., Jia, Y.C., Cui, K., Li, N., Zheng, Z.Y., Wang, Y.Z., Yang, X.B., 2005. Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 434, 894–898.

Lima, B., Forrester, T., Hess, D.T., Stamler, J.S., 2010. S-nitrosylation in cardiovascular signaling. Circ. Res. 106, 633–646.

Liu, S., Mansour, M.N., Dillman, K.S., Perez, J.R., Danley, D.E., Aeed, P.A., Simons, S.P., LeMotte, P.K., Menniti, F.S., 2008. Structural basis for the catalytic mechanism of human phosphodiesterase 9. PNAS 105, 13309–13314.

Loewi, O., 1921. Uber humorale ubertragbarkeit der Herznervenwirkung. Pflugers Arch. 189, 239–242.

Logothetis, D.E., Kurachi, Y., Galper, J., Neer, E.J., Clapham, D.E., 1987. The βγ subunits of GTP-binding proteins activate the muscarinic K1 channel in heart. Nature 325, 321–326.

Lugnier, C., 2006. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol. Ther. 109, 366–398.

Lukas, R.J., Changeux, J.-P., LeNovere, N., Albuquerque, E.X., Balfour, D.J.K., et al., 1999. International Union of Pharmacology; XX; Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharm. Rev. 51, 397–401.

Luscher, C., Slesinger, P.A., 2010. Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. Nat. Rev. Neurosci. 11, 301–315.

MacFarland, R.T., Zelus, B.D., Beavo, J.A., 1991. High concentrations of a cGMP-stimulated phosphodiesterase media ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. JBC 266, 136–142.

Magleby, K.L., Pallotta, B.S., 1981. A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. J. Physiol. 316, 225–250.

Mak, D.P., McBride, S., Foskett, J.K., 1998. Inositol 1,4,5-trisphosphate activation of inositol tris-phosphate receptor Ca^{2+} channel by ligand tuning to Ca^{2+} inhibition. PNAS U.S.A. 95, 15821–15825.

Maus, A.D., Pereira, E.F., Karachunski, P.I., Horton, R.M., Navaneetham, D., Macklin, K., Cortes, W.S., Albuquerque, E.X., Conti-Fine, B.M., 1998. Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. Mol. Pharmacol. 54, 779–788.

McGranahan, T.M., Patzlaff, N.E., Grady, S.R., Heinemann, S.F., Booker, T.K., 2011. $\alpha4\beta2$ nicotinic acetylcholine receptors on dopaminergic neurons mediate nicotine reward and anxiety relief. J. Neurosci. 31, 10891–10902.

Meurs, H., Dekkers, B.G.J., Maarsingh, H., Halayko, A.J., Zaagsma, J., Gosens, R., 2013. Muscarinic receptors on airway mesenchymal cells: novel findings for an ancient target. Pulm. Pharmacol. Ther. 26, 145–155.

Miyakawa, T., Mizushima, A., Hirose, K., Yamazawa, T., Bezprosvanny, I., Kurosaki, T., Iino, M., 2001. Ca^{2+}-sensor region of IP_3 receptors controls intracellular Ca^{2+} signalling. EMBO J. 20, 1674–1680.

Miyazawa, A., Fujiyoshi, Y., Unwin, N., 2003. Structure and gating mechanism of the acetylcholine receptor pore. Nature 423, 949–955.

Mukherjee, S., Trice, J., Shinde, P., Willis, R.E., Pressley, T.A., Perez-Zoghbi, J.F., 2013. Ca^{2+} oscillations, Ca^{2+} sensitization, and contraction activated by protein kinase C in small airway smooth muscle. J. Gen. Phys. 141, 165–178.

Murad, F., Mittal, C.K., Arnold, W.P., Katsuki, S., Kimura, H., 1978. Guanylate cyclase activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. Adv. Cycl. Nucleotide Res. 9, 145–158.

Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A., Fujiyoshi, Y., 2000. Structural determinants of water permeation through aquaporin-1. Nature 407, 599–605.

Nahorski, S.R., Tobin, A.B., Willars, G.B., 1997. Muscarinic M3 receptor coupling and regulation. Life Sci. 60, 1039–1045.

Narayanam, D., Adelbiyi, A., Jaggar, J.H., 2012. Inositol trisphosphate receptors in smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol. 302, H2190–H2210.

Neher, E., Sakman, B., 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature 260, 799–802.

Nichols, C.G., Lopatin, A.N., 1997. Inward rectifier potassium channels. Annu. Rev. Physiol. 59, 171–179.

Nikolaev, V.O., Gambaryan, S., Engelhardt, S., Walter, U., Lohse, M.J., 2005. Real-time monitoring of the PDE2 activity in life cells: hormone stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. J. Biol. Chem. 280, 1716–1719.

Nioche, R., Berka, V., Vipond, J., Minton, N., Tsai, A.L., Raman, C.S., 2004. Femtomolar sensitivity of a NO sensor from Clostridium botulinum. Science 306, 1550–1553.

Nys, M., Kesters, D., Ulens, C., 2013. Structural insights into Cys-loop receptor function and ligand recognition. Biochem. Pharmacol. 86, 1042–1053.

Osborne, B.W., Wu, J., McFarland, C.J., Nickl, C.K., Sankaran, B., Casteel, D.E., Woods, V.L., Kornev, A.P., Taylor, S.S., Donstmannn, W.R., 2011. Crystal structure of cGMP-dependent protein kinase reveals novel site of interchain communication. Structure 19, 1317–1327.

Palmer, R.M., Ferrige, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Pandit, J., Forman, M.D., Fennell, K., Dillman, K.S., Menniti, F.S., 2009. Mechanism for the allosteric regulation of phosphodiesterase 2A deduced from the X-ray structure of a near full-length construct. PNAS U.S.A. 106, 18225–19182.

Peel, S.E., Liu, B., Hall, I.P., 2008. ORAI and store-operated calcium influx in human airway smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 38, 744–749.

Pellicena, P., Karow, D.S., Boon, E.M., Marietta, M.A., Kuriyan, J., 2004. Crystal structure of an oxygen-heme domain related to soluble guanylate cyclases. PNAS 101, 12854–12859.

Perez, J.F., Sanderson, M.J., 2005. The frequency of calcium oscillations induced by 5-H, ACh and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J. Gen. Physiol. 125, 535–553.

Postea, O., Biel, M., 2011. Exploring HCN channels as novel drug targets. Nat. Rev. Drug Discov. 10, 903–914.

Qian, J., Fulton, D., 2013. Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Front. Physiol. 4 article 347.

Rang, H.P., Ritter, J.M., 1970. On the mechanism of desensitization at cholinergic receptors. Mol. Pharmacol. 6, 357–382.

Rapoport, R.M., Murad, F., 1983. Agonist-induced endothelium-dependent relaxations in rat thoracic aorta may be mediated through cGMP. Cric. Res. 8, 37–44.

Redka, D.S., Pisterzi, L.F., Wells, J.W., 2008. Binding of orthosteric ligands to the allosteric site of the M2 muscarinic cholinergic receptor. Mol. Pharmacol. 74, 834–843.

Roscioni, S.S., Maarsingh, H., Elzinga, C.R.S., Schuur, J., Menzen, M., Halayko, A.J., Meurs, H., Schmidt, M., 2011. Epac as a novel effector of airway smooth muscle contraction. J. Cell Mol. Med. 15, 1551–1563.

Ruth, P., Wang, G.X., Boekhoff, I., May, B., Pfeifer, A., Penner, R., Korth, M., Breer, H., Hofmann, F., 1993. Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production. Proc. Natl. Acad. Sci. U.S.A. 90, 2623–2627.

Salpeter, S., Buckley, N., Ormiston, T., Salpeter, E., 2006. Meta-analysis: effect of long-acting beta-agonists on severe asthma exacerbations and asthma-related deaths. Ann. Intern. Med. 144, 904–912.

Sauer, K., Cooke, M.P., 2010. Regulation of immune cell development through soluble inositol-1,3,4,5-tetrakisphosphate. Nat. Rev. Immunol. 10, 257–271.

Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., Wang, G.X., Allescher, H.D., Korth, M., Wilm, M., Hofmann, F., Ruth, P., 2000. Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. Nature 404, 197–201.

Schoeffter, P., Lugnier, C., Demesy-Waeldele, F., Stoclet, J.C., 1987. Role of cyclic AMP- and cyclic GMP-phosphodiesterases in the control of cyclic nucleotide levels and smooth muscle tone in rat aorta. A study with selective inhibitors. Biochem. Pharmacol. 36, 3965–3972.

Schoen, C., Bachman, G., 2009. Sildenafil citrate for female sexual arousal disorder: a future possibility? Nat. Rev. Urol. 6, 216–222.

Sellin, L.C., McArdle, J.J., 1994. Multiple effects of 2,3-butanedione monoxime. Pharmacol. Toxicol. 74, 305–313.

Shaughnessy, J.D., Largaespada, D.A., Tian, E., Fletcher, C.F., Cho, B.C., Vyas, P., Jenkins, N.A., Copeland, N.G., 1999. Mrvi1, a common MRV integration site in BXH2 myeloid leukemias, encodes a protein with homology to a lymphoid-restricted membrane protein Jaw1. Oncogene 18, 2069–2084.

Shaul, P.W., Smart, E.J., Robinson, L.J., German, Z., Yuhanna, I.S., 1996. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. Biol. Chem. 271, 6518–6522.

Shesely, E.G., Maeda, N., Kim, H.S., Desai, K.M., Krege, J.H., Laubach, V.E., Sherman, P.A., Sessa, W.C., Smithies, O., 1996. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. PNAS U.S.A. 93, 13176–13181.

Sixma, T.K., Smith, A.B., 2003. Acetylcholine binding protein (AChBP): a secreted glial protein that provides a high resolution model for the extracellular domain of pentameric ligand-gated ion channels. Annu. Rev. Biophys. Biomol. Struct. 32, 311–334.

Smith, A.B., Syed, N.I., Schaap, D., van Minnen, J., Klumperman, J., Kits, K.S., Lodder, H., van der Schors, R.C., van Elk, R., Sorgedrager, B., Brejc, K., Sixma, T.K., Geraerts, W.P., 2001. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. Nature 411, 261–268.

Sobotta, J., Becher, H., 1975. Atlas of human anatomy. In: Visceral Anatomy. 9th English Edition, vol. 2. Urban and Schwartzenberg.

Stiber, J.A., Rosenberg, P.B., 2011. The role of store-operated calcium influx in skeletal muscle signaling. Cell Calcium 49, 341–349.

Tao, S., Yamazaki, D., Lomazaki, S., Zhao, C., Iida, T., Kakizawa, S., Imaizumi, Y., Takeshima, H., 2013. Facilitated hyperpolarization signaling in vascular smooth muscle-overexpressing TRIC-A channels. J. Biol. Chem. 288, 15581–15589.

Tashkin, D.P., Celli, B., Senn, S., Burkhart, D., Kesten, S., Menjoge, S., et al., 2008. A 4-year trial of tiotropium in chronic obstructive pulmonary disease. N. Engl. J. Med. 359, 1543e54.

Unglaub-Silverthorn, D., 2010. Human Physiology: An Integrated Approach, fifth ed. Pearson Benjamin Cummings.

Unwin, N., Toyoshima, C., Kubalek, E., 1988. Arrangement of the acetylcholine receptor subunits in the resting and desensitized states, determined by cryoelectron microscopy of crystallized Torpedo postsynaptic membranes. J. Cell Biol. 107, 1123–1138.

Unwin, N., Miyazawa, A., Li, J., Fujiyoshi, Y., 2002. Activation of the nicotinic acetylcholine receptor involves a switch in conformation of the α subunits. J. Mol. Biol. 319, 1165–1176.

Unwin, N., 1995. Acetylcholine receptor channel imaged in the open state. Nature 373, 37–43.

Unwin, N., 2005. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J. Mol. Biol. 346, 967–989.

Vasudevan, N.T., Mohan, M.L., Gowani, S.K., Naga-Prasad, S.V., 2011. Regulation of β-adrenergic receptor function: an emphasis on receptor resensitization. Cell Cycle 10, 3684–3691.

Walker, J.K., Penn, R.B., Hanania, N.A., Dickey, B.F., Bond, R.A., 2011. New perspectives regarding β2-adrenoceptor ligands in the treatment of asthma. Br. J. Pharmacol. 163, 18e28.

Wang, Y.Z., Zheng, Y.M., 2011. Molecular expression and functional role of canonical transient receptor potential channels in airway smooth muscle cells. Adv. Exp. Med. Biol. 704, 731–747.

Whorton, M.R., MacKinnon, R., 2013. X-ray structure of the mammalian GIRK2-βγ G-protein complex. Nature 498, 190–197.

Wickman, K.D., Iniguez-Lluhl, J.A., Davenport, P.A., Taussig, R., Krapivinsky, G.B., Linder, M.E., Gilman, A.G., Clapham, D.E., 1994. Recombinant G-protein beta-gamma subunits activate the muscarinic-gated atrial potassium channel. Nature 368, 255–257.

Williams, O.W., Sharafkhaneh, A., Kim, V., Dickey, B., Evans, C.M., 2006. Airway mucus. Am. J. Resp. Cell Mol. Biol. 34, 527–536.

Wooldridge, A.A., MacDonald, J.A., Erdodi, F., Ma, C.Y., Borman, M.A., Hartshorne, D.J., Haystead, T.A.J., 2004. Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of serine 695 in response to cyclic nucleotides. J. Biol. Chem. 279, 34496–34504.

Xia, H.J., Yang, G., 2005. Inositol 1,4,5-trisphosphate 3-kinases: functions and regulations. Cell Res. 15, 83–91.

Xiao, R.P., Tomhave, E.D., Wang, D.J., Ji, X., Boluyt, M.O., Cheng, H., Lakatta, E.G., Koch, W.J., 1998. Age-associated reductions in cardiac beta1- and beta2-adrenergic responses without changes in inhibitory G proteins or receptor kinases. J. Clin. Invest. 101, 1273–1282.

Zhang, K.Y.J., Card, G.L., Suzuki, Y., Artis, D.R., Fong, D., Gillette, S., Hsieh, D., Neiman, J., West, B.L., Zhang, C., Milburn, M.V., Kim, S.-H., Schlessinger, J., Bollag, G., 2004. A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. Mol. Cell 15, 279–286.

Zieba, B.J., Artamonov, M.V., Jin, L., Momotani, K., Ho, R., Franke, A.S., et al., 2011. The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. J. Biol. Chem. 286, 16681–16692.

CHAPTER

5

Sensory Signal Processing; Visual Transduction and Olfaction

VISUAL TRANSDUCTION

> *Do not the Rays of Light in falling upon the bottom of the Eye excite Vibrations in the Tunica retina? Which Vibrations, being propagated along the solid Fibres of the optick Nerves into the Brain, cause the sense of seeing.* ***Sir Isaac Newton, Opticks (1704) p. 345.***

The visual system provides an exceptional opportunity to investigate the transduction mechanism of a G-protein-coupled receptor (GPCR) at the level of a single molecule. The molecule is the photoreceptor-cell pigment rhodopsin. This consists of the protein, opsin, to which is bound the photosensitive compound 11-*cis*-retinal. The stimulus is light and the second messengers are cyclic GMP and Ca^{2+} (Lagnado and Baylor, 1992).

OCULAR PHOTORECEPTOR CELLS

In the course of evolution, two major classes of ocular (eye) cells have become specialized in phototransduction: ciliary photoreceptor cells, typified by vertebrate rods and cones, and the microvillar, or rhabdomeric, photoreceptor cells of many invertebrate groups, including arthropods and most mollusks. Ciliary and microvillar photoreceptor cells coexist in most major divisions of the animal kingdom (ciliary photoreceptor cells exclusively in the retina and microvillar photoreceptor cells in the retina and elsewhere) and because of this they are believed to have arisen and evolved independently prior to the separation of vertebrates and invertebrates in the Precambrian period (550 million years ago (Arendt, 2003)). Although all known ocular photoreceptor cells employ 11-*cis*-retinal and opsins to absorb the incident light energy and employ a G-protein-coupled signaling cascade to transduce this into changes in conductance in the

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plasma membrane, the signaling pathways are quite distinct. In first instance, we deal with vertebrate photoreceptors and then we will briefly show how flies differ from us.

The ciliary photoreceptor cells, their connecting neurons, and associated pigment epithelial cells constitute the retina. The retina is a part of the central nervous system derived from the neural tube. It arises from optic vesicles which fold back in upon themselves to form the optic cup. The inside of the cup becomes the retina and the outside remains a single monolayer of epithelium, known as the retinal pigment epithelium (Figure 5-1). Initially both walls of the optic cup are one cell thick, but the cells of the inner layer divide many cells thick and develop into, from inside to outside, ganglion-, amacrine-, bipolar-, and horizontal cells and finally give rise to photoreceptor cells of two types: rods and cones. As a consequence, light first traverses the neurons before reaching the rods and cones and is then absorbed by the retinal pigment epithelium (loaded with melanin- and lipofuscin-granules). This epithelium plays an important role in the recycling of retinal, the photoreceptor ligand, and prevents light being scattered back into the photoreceptor layer. A failure to synthesize melanin, associated with the inherited conditions described as albinism, causes problems associated with bright lights and glare.

Beyond these difficulties in albinism, the fovea (central region of the retina responsible for visual acuity) may also fail to develop properly, so that the eye cannot produce sharp images well.

Cones and rods

The photoreceptor cells, both rods and cones, consist of four parts, starting from the inside, a synaptic terminal, a cell body with the nucleus, an inner- and an outer-segment (Figure 5-2). The inner and outer segments are connected by an axoneme, a radial spoke structure composed of microtubules which we also find in cilia of epithelial cells in the lung for instance. The axoneme serves as a physical support and is instrumental in the transport of vesicles derived from the Golgi and destined for the outer segment (Deretic and Wang, 2012). The plasma membrane generates protrusions at the interface between the inner- and outer-segment and these develop into a stack of membranes which is densely packed with photoreceptors (rhodopsin). Cones differ from rods not just because they have a cone-shaped outer segment but also with respect to the membrane stack: the protrusions do not fuse into

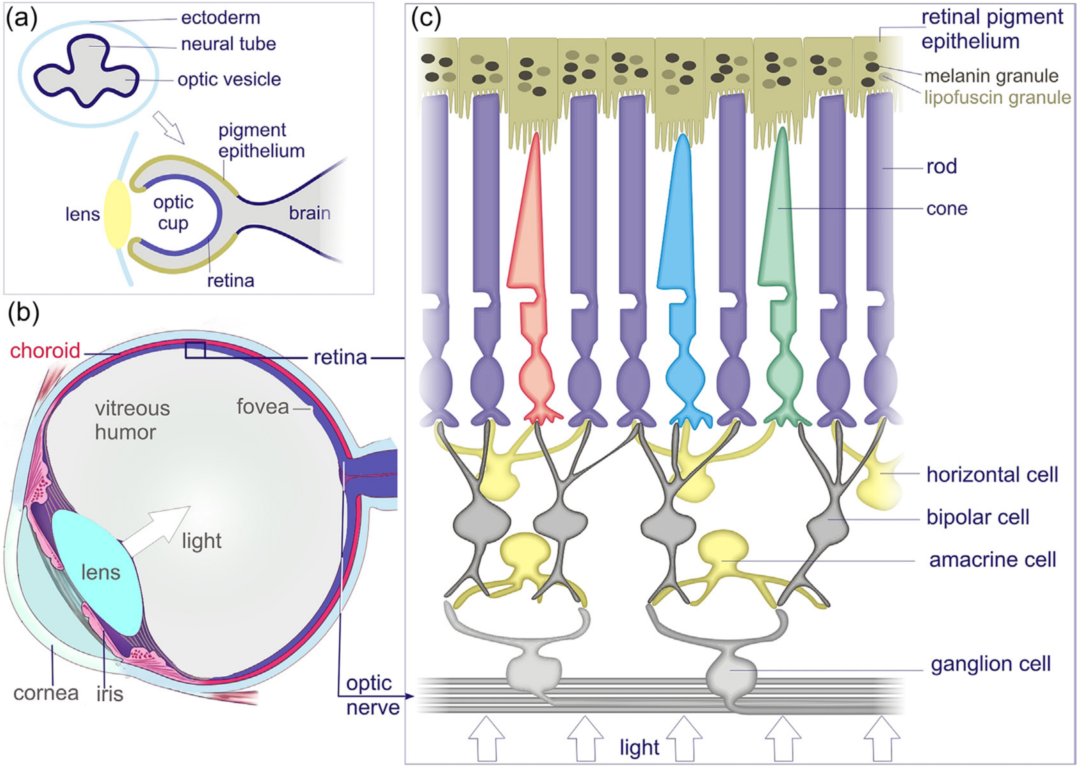


FIGURE 5-1 Eye development and composition of the human retina. (a) The eye develops from an optic vesicle, derived from the neural tube. It converts into an optic cup of which the cells on the inside develop into a neuronal network of multiple layers plus photoreceptor cells. The outer layer develops into pigment epithelium. The lens and the cornea are derived from cells of the ectoderm. (b) Cross section of the human eye. (c) The human retina comprises the optic nerve, ganglion-, amacrine-, bipolar-, and horizontal cells. These are connected, directly or indirectly, with the photoreceptor cells (rods and cones). The retinal pigment epithelium surrounds the retina. It contains two types of pigments, melanin- and lipofuscin-granules, which are put in place to absorb light so as to prevent scattering. Notice that the light first traverses the neuronal network before reaching the photoreceptor cells.

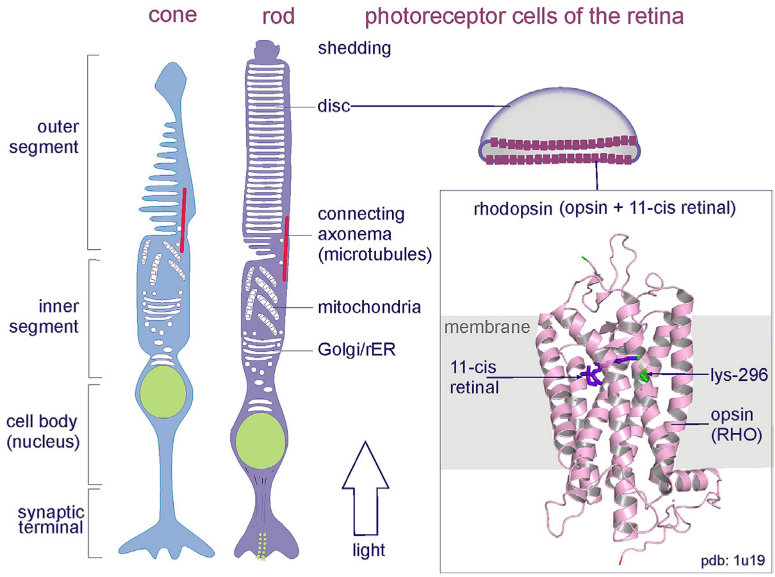


FIGURE 5-2 Photoreceptor cells and photoreceptors of the human retina. Cones and rods are made of four segments, a synaptic terminal, a cell body, an inner segment, and an outer segment. The inner- and outer-segment are connected via an axoneme (microtubule structure found in cilia). The outer segment makes numerous membrane disks, each containing large numbers of photoreceptor proteins, rhodopsin. Rhodopsin is the photoreceptor cell visual pigment. It is a seven transmembrane-spanning protein, named opsin, bound to a light-sensitive chromophore, 11-*cis*-retinal.

intracellular discs. The array of membrane discs serves to create a large membrane surface, allowing the cells to express very large numbers of photoreceptors. A single human rod cell contains on average 10^8 photoreceptors (membrane receptors are normally expressed in the order of 10^4/cell) (Daemen, 1973). The need for such vast amounts of photoreceptors reflects the need for sensitivity. As we have learned from pharmacological studies, increasing receptor numbers increases the sensitivity of the system (return to Chapter 2, Figure 2-11). Finally, the membrane discs are removed through a process of membrane shedding at the side of the pigment epithelium (Young, 1967).

Visual pigment: opsins and their chromophore

The molecular composition of the photoreceptor comprises a seven transmembrane protein named opsin (member of the GPCR), and a covalently bound chromophore, 11-*cis*-retinal. Together, protein and chromophore, they constitute the visual pigment. The human retina has four different types of pigments as reflected by four different types of opsins: rods express just one, RHO, but cones may express ON1SW, OPN1MW, or OPN1LW (short-, medium-, or long-wavelength opsin respectively). While the chemical structure of the chromophore (11-*cis*-retinal) is identical for all of them (with the exception that the Schiff base linkage is unprotonated in the case of ON1SW), the absorption spectrum varies between the different visual pigments and that is why we can discriminate colors. The three different cone opsins tune the absorption spectrum of the attached retinal to sense either purple/blue, green, or red light (more or less primary colors). The unique light absorption spectra are determined by specific interactions between the 11-*cis*-retinal and surrounding amino acids of the opsin (see Figure 5-3). Mutations of these amino acids cause changes in the absorption spectra and naturally occurring mutations are indeed instrumental in the phenomenon of color blindness (Imamoto and Shichida, 2013).

Besides different absorption spectra, rods and cones also make a difference with respect to light sensitivity. Rods are responsive to a single photon and operate under low light conditions (scotopic vision), whereas cones require higher light intensity (photopic vision). This explains why color is lost when objects are viewed in dim light. The human retina contains about 120 million rods and about 6 million cones. Subsequent discussion will concentrate on rod cells.

NOMENCLATURE

The nomenclature for opsins is somewhat confusing. The name rhodopsin (rod opsin + 11-*cis*-retinal) comes from Greek and combines *rhodon,* rose, and *opsis,* vision. Rhodopsin has a purple appearance (visual purple pigment of the retina) and this was first reported by the German physiologist Franz Christian Boll in 1876. He also observed that the rod pigment had a tendency to fade in the presence of illumination (so-called photobleaching). Wilhelm Kühne published one of the first articles on the photochemical theory, establishing a link between visual purple and vision in light of low intensity (Kühne, 1878). He showed that the chromophore was distinct from those present in egg yolk (lutein or β-carotene-diol). Kühne is also known for discovering trypsin and myosin and for coining the phrase "enzyme." The cone opsins bound to 11-*cis*-retinal are sometimes referred to as iodopsins and the apoproteins (lacking the ligand) are named photopsins. The three iodopsins are also named cyanolabe (blue/purple, shortwave), chlorolabe (green, medium wave), and erythrolabe (red, long wave).

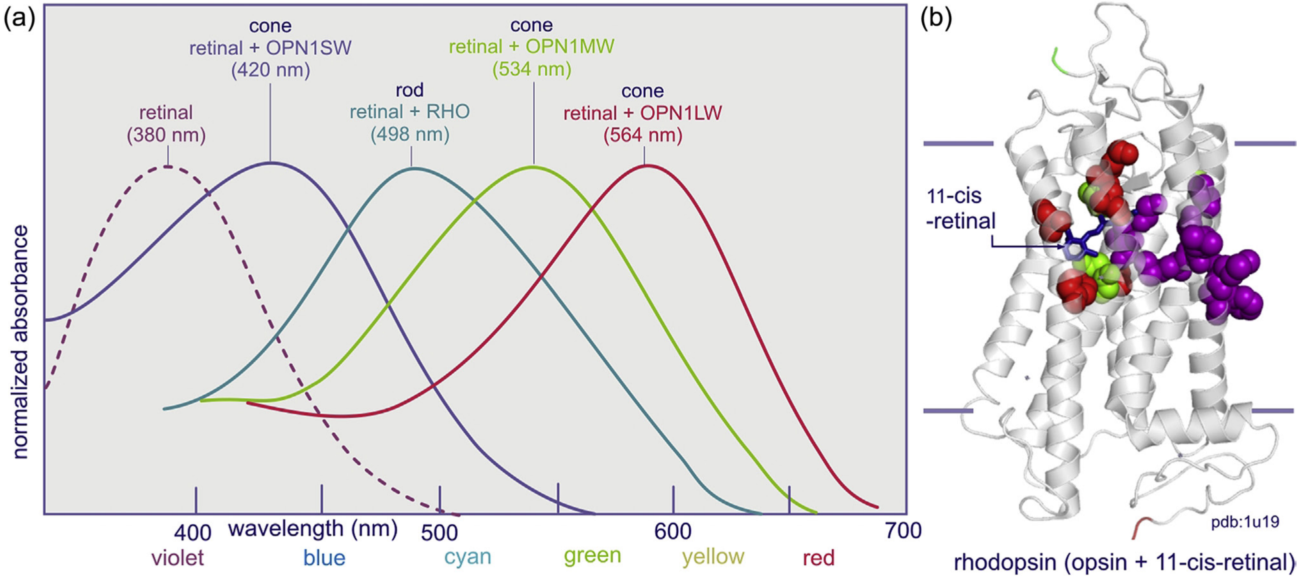


FIGURE 5-3 The absorption spectra of the different rhodopsins. (a) All photoreceptor cells contain the same chromophore, *cis*-retinal, but it is embedded in four different proteins, the opsins. We recognize three different cone opsins (ON1SW, OPN1MW, and OPN1LW), which operate in bright light conditions and allow color discrimination (primary colors). Their wavelength absorption, in the presence of retinal, reaches a maximum at 420, 534, and 564 nm, ranging from shortwave blue purple to longwave red light. Rods have only one opsin (RHO) and when bound to *cis*-retinal, absorption peaks at 498 nm. (b) Opsin structure showing the positions of the amino acids that determine, in part, the absorption spectra of the different retinal pigments. Those colored in purple establish a wavelength peak at 420 nm, those in green a peak at 534, and those in red a peak at 564 nm. Pdb: 1u19.

Scotopic vision is the vision of the eye under low light conditions. The term comes from Greek *skotos* meaning *darkness* and *-opia* meaning *a condition of sight*.

Light is defined by its wave properties—frequency (ν) and wavelength (λ) where the velocity $c = \nu \times \lambda$. The intensity of a beam of light is the rate at which energy is delivered per unit area. It is measured in W/m^2. Light also possesses particle properties. The quanta are called photons and the energy (in J) of a single photon is given by $E = h \times \nu$, where h is Planck's constant (6.626×10^{-34} J s).

PHOTORECEPTOR MECHANISMS

We know more about signaling through vertebrate photoreceptors than through any of the other GPCRs (or 7TM) interacting with heterotrimeric GTP-binding proteins. However, a warning is in order. It should not be imagined that what we learn here is a general reflection of GPCR mechanisms. Almost everything that happens in vertebrate phototransduction is the very converse of the normal sequence of events by which ligands activate receptors and receptors signal the generation of second messengers and then their interactions with effectors. This is a world turned upside down. For a start, 11-*cis*-retinal is a receptor ligand that is already bound covalently before receipt of the light signal. It acts as an inverse agonist, and as we discussed in Chapter 2 and 3, an inverse agonist prevents spontaneous receptor activation. After light-induced photoisomerization, where retinal transforms from an 11-*cis*- to an all-*trans* conformation, it detaches from the photoreceptor. Second, the light signal does not cause the production of second messenger, cyclic GMP, but instead leads to its breakdown. Lastly, whereas the seven membrane-spanning receptors discussed in previous chapters generally cause an increase in intracellular free Ca^{2+}, photoreceptors are coupled to a process which reduces intracellular free Ca^{2+} (and as a consequence reduce the secretion of neurotransmitter).

By contrast, in the invertebrate photoreceptor system we appear to be on rather more familiar ground. Here, the excitation of rhodopsin is coupled mostly through Gαq to the activation of PLCβ and elevation of cytosol Ca^{2+} (discussed later on Hardie and Postma (2008)).

Photoisomerization of 11-*cis*-retinal causes receptor activation

The chromophore, 11-*cis*-retinal, is coupled through its aldehyde group to the α-amino acid group of a lysine in the center of the transmembrane segment of rhodopsin, as a protonated Schiff base (in the case of the cone-opsin ON1SW, the bond is not protonated). The chromophore stabilizes the receptor in an inactive conformation, meaning that it prevents rhodopsin from interacting with downstream effectors. This is why 11-*cis*-retinal is considered an inverse agonist. Its role as an inverse agonist makes much sense in view of the observation that GPCRs signal spontaneously (return to Chapter 3, Section "Adrenoceptor agonists, antagonists, and inverse agonists"). Given the enormous number of rhodopsin proteins in each rod, about 10,000 times higher than average expression levels of cell-membrane receptors, spontaneous signaling would be substantial and thus would give rise to spurious "dark vision." When a photon hits the C11 or C12 of 11-*cis*-retinal, thereby, pushing one electron in an energetically higher orbital, the double bond between the two carbons transiently disassembles and the molecule rotates from a *cis*-to *trans*-conformation. This is followed by hydrolysis of the Schiff base, thereby, forming free all-*trans*-retinal (which is rapidly transformed into all-*trans*-retinol by replacing the aldehyde group with an alcohol) (Figure 5-4). All-*trans*-retinol leaves the binding pocket and inserts into the disc membrane. In this process, the absorption spectrum of rhodopsin (initially purple) goes from photo, batho, blue-shifted intermediate, lumi, meta I to meta II (yellow) in a timescale of milliseconds (Figure 5-5(b)). The receptor stabilizes in the metarhodopsin II state and we may consider this the "active receptor" (also known as photoexcited rhodopsin).

The above-described spectral changes represent important conformational changes of the receptor of which fluctuations of helices H5 and H6 are most notable (Struts et al., 2011; Choe et al., 2011). Together these fluctuations expose, in first instance, recognition sites for the α-subunit of transducin (Gαt) on the cytoplasmic surface, very much in the same way as adrenaline creates a binding pocket for Gαs (Rasmussen et al., 2011; Nygaard et al., 2013). Figure 5-5(a) illustrates rhodopsin with the characteristic seven transmembrane helices (H1–7) viewed from the side and from the cytoplasm. Apart from a number of intramolecular rearrangements (not shown), metarhodopsin II is characterized by the extension of H5 and the outward shift of the cytoplasmic segment of H6. Both changes create a cavity which fits the α-5 helix of Gαt (human genome name GNAT). Gαt is part of a heterotrimeric G-protein, including two other subunits Gβ and Gγ, and collectively they constitute "transducin" (Wheeler et al., 1977; Pappone et al., 1982).

Binding of Gαt to metarhodopsin II results in the exchange of GDP for GTP, the receptor acts as a guanine-exchange factor, and this leads to subsequent changes in the switch region of the protein (for detail of the exchange

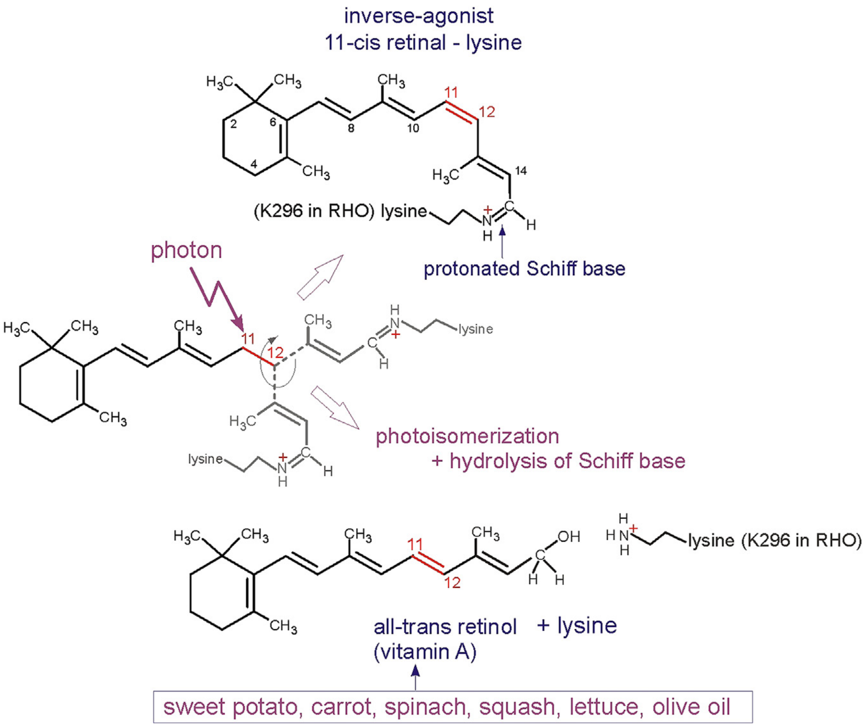


FIGURE 5-4 Structure of retinal inside the opsin molecule. Retinal is inserted into opsin in its 11-*cis* conformation. It binds covalently to lysine (K296 in human RHO) by means of a protonated Schiff base. When a photon hits carbon 11 or -12, one of the two electrons in the pi-orbit moves temporally to a higher-energy orbit and this breaks the double bond. The molecule now rotates around carbon-12 until the electron returns to its original orbit and reestablishes the double bond. The molecule normally ends up in an all-*trans* configuration. The Schiff base is then hydrolyzed leading to the formation of a free all-*trans*-retinol which will leave the binding pocket. All-*trans*-retinol represents one of the many molecular entities which we collectively refer to as vitamin A (abundantly present in sweet potato, carrot, spinach, squash, lettuce, or olive oil).

reaction, we refer to Chapter 3, Figure 3-7). As a consequence, Gαt dissociates from Gβγ and is now ready to interact with an inhibitory subunit (γ) of phosphodiesterase-6 (PDE6) (Figure 5-6; Liebman and Pugh, 1979). Normally, tens of transducins interact with one metarhodopsin II and each transducin binds to a single inhibitory subunit. Retinal PDE6 forms a dimer comprising two catalytic subunits, PDE6A and PDE6B, both anchored to the membrane by a lipid chain (return to Chapter 4, Figure 4-15). Regulation occurs through a third subunit, PDE6γ (PDE6G). It is a structurally disordered protein which makes contact with the GAF-A domain (N-terminal sequence of PDE) and the catalytic site (C-terminal sequence). It prevents access of substrate. Active Gαt sequesters the γ-subunit, holding it away from the catalytic site.

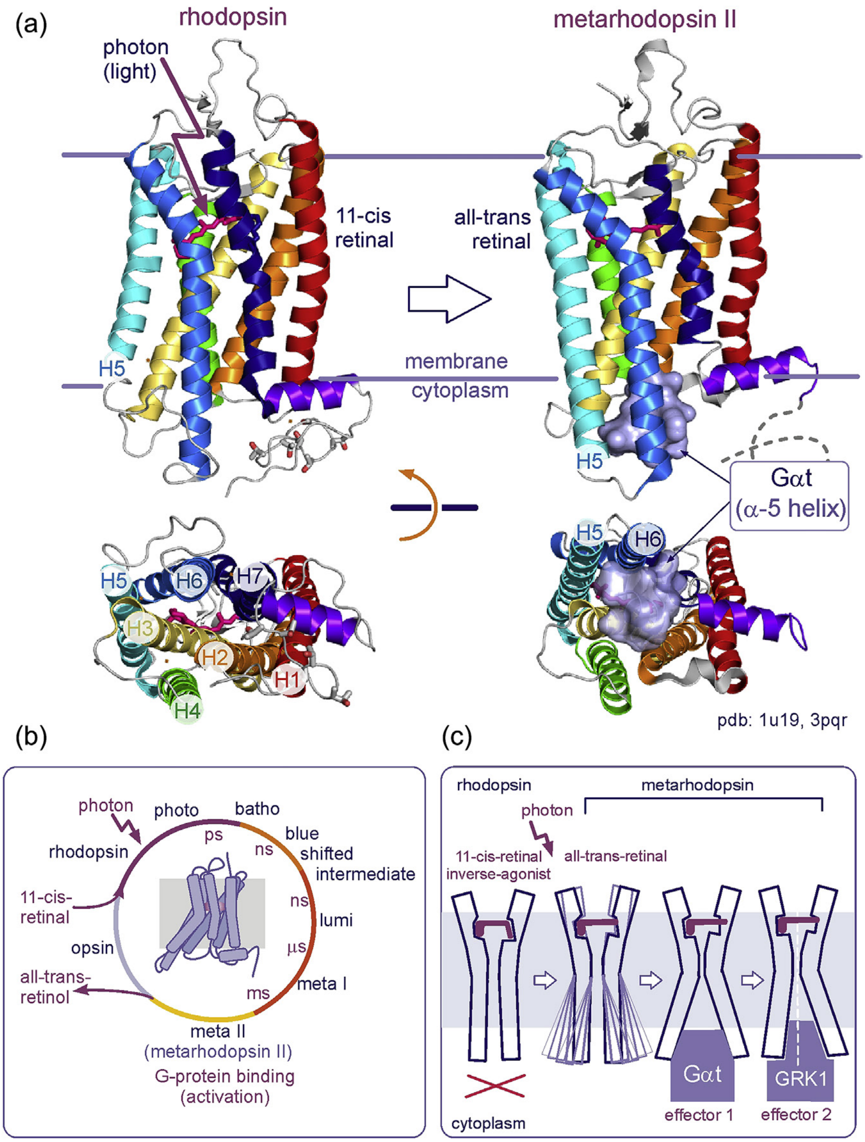


FIGURE 5-5 Molecular structure of rhodopsin and metarhodopsin II. (a) Rhodopsin, opsin + 11-*cis*-retinal, is a prototypical G-protein-coupled receptor (or 7TM protein). The membrane helices are colored from red to purple. 11-*cis*-retinal poses in the transmembrane segment of the receptor where it is covalently bound, in the form of a protonated Schiff base, to lysine (K296 in human rhodopsin). Upon absorption of a photon, 11-*cis*-retinal isomerizes (photoisomerization) into all-*trans*-retinal which after hydrolysis of the Schiff base becomes all-*trans*-retinol. The isomerization process leads to important structural changes, of which the extension of helix-5 and the outward movement of H6 are most prominent. Both changes create a binding pocket that fits the α-5 helix of Gαt (the Gα-subunit of transducin). The binding stabilizes the receptor in its active state. (b) The photoisomerization process is accompanied by very rapid changes in the spectral properties of rhodopsin. The changes go from photo, batho, blue-shifted intermediate, lumi, meta I to metarhodopsin II. The protein stabilizes in this configuration and interacts with Gαt.

RETINAL IS DERIVED FROM VITAMIN A

As a postdoctoral researcher, in 1933, George Wald discovered that vitamin A was a component of the retina. His further experiments showed that when rhodopsin was exposed to light, it yielded the protein opsin and a compound containing vitamin A. This suggested that vitamin A was essential in retinal function. He won a share of the 1967 Nobel Prize in Physiology or Medicine with Haldan Keffer Hartline and Ragnar Granit "for their discoveries concerning the primary physiological and chemical visual processes in the eye."

Vitamin A is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids, among which beta-carotene is the most important. Sweet potato, carrot, spinach, squash, lettuce, and olive oil are rich in vitamin A. Apart from its primordial role in vision, it is important for growth and development (retinoic acid) and for the maintenance of the immune system.

Signaling through cGMP-PDE is coupled with changes in synaptic glutamate release

Sequestration of the inhibitory γ-subunit of PDE6 renders the catalytic site accessible to substrate and cGMP is transformed into GMP (hydrolysis of the cyclic bond, return to Chapter 4, Figure 4-17) (Granovsky et al., 1997; Liu et al., 1998; Arshavsky et al., 2002; Slep et al., 2001). The loss of second messenger causes an abrupt closure of the cGMP-gated CNGA1 channels. A reduction in the inward current brings about a lowering of the membrane potential in the range of 1–30 mV (depending on the light intensity). With strong light, the potential lowers to a minimum of −73 mV, which is near the equilibrium potential for K^+. Inward and outward currents are compensated by outward- and inward-operating pumps and exchangers (see Figures 5-6 and 5-7).

Hyperpolarization of the rod plasma membrane is translated into a reduced conductivity of the voltage-sensitive Ca^{2+} channel CACNA1F (Ca_v1.4). These are located in the synaptic terminal near the docking site of secretion vesicles loaded with glutamate (active zone of presynaptic membrane). High levels of Ca^{2+}, in the order of 100–400 μM, bring about a constant release of neurotransmitter by binding to synaptotagmin and

◀ (c) The ligand (or chromophore), 11-*cis*-retinal, acts as an inverse agonist; it prevents "intrinsic" or "spontaneous" activation of the receptor. Isomerization removes the inhibitory constraint allowing the protein to adopt different configurations (ending in the metarhodopsin II state). This opens the way for Gαt (transducin complex) to attach to and stabilize the receptor in its active state so that exchange of GDP for GTP follows. The activated transducin propagates the light signal into the cell. Another protein, G-protein-regulated kinase-1 can also interact with metarhodopsin II. It plays a role in inhibiting subsequent interactions with transducins.

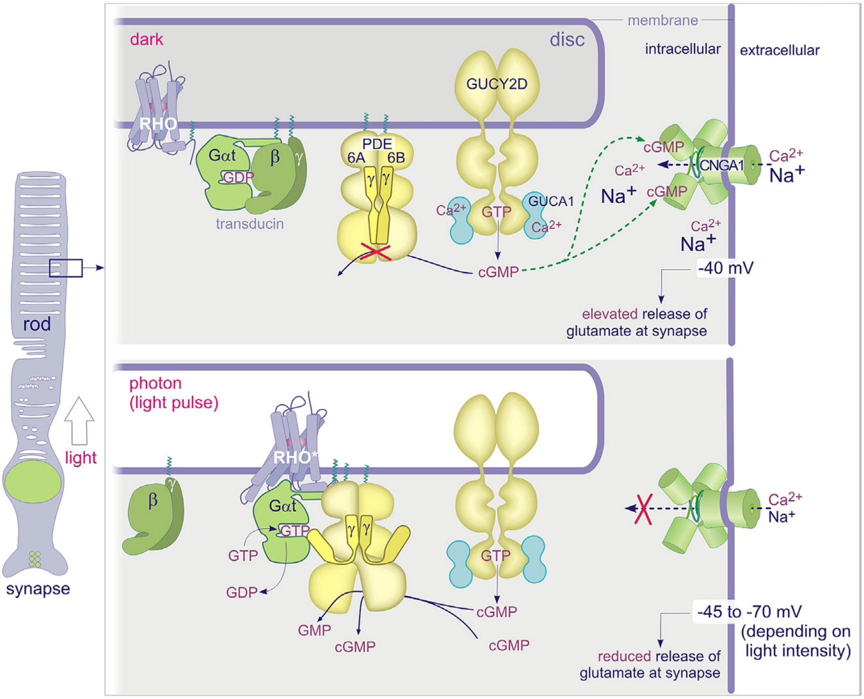


FIGURE 5-6 Phototransduction in rods. In the dark state, transducin is in its inactive GDP-bound state, PDE6 is also inactive, whereas guanylyl cyclase (GUCY2D) produces cGMP at a low rate (activity constraint by Ca^{2+} bound to GUCA1). The intracellular cGMP binds and increases conductivity of the cyclic nucleotide-gated cation channel CNGA1, leading to a steady inward Na^+ current with a minor Ca^{2+} component. The leakage for Na^+ and Ca^{2+} raises the resting membrane potential to an unusual level of −40 mV. Following a light pulse, isomerization of 11-*cis*-retinal causes the formation of metarhodopsin II (RHO*) which in turn causes the binding of transducin. Subsequent exchange of GDP for GTP activates Gαt and this leads to the binding of PDE6γ, an inhibitory subunit of PDE6. Sequestration of the inhibitory subunit results in the transformation of cGMP into GMP and the subsequent loss of cGMP results in a closure of the cyclic nucleotide CNGA1 channel. The reduced inward leakage lowers the membrane potential to −70 mV (due to an initially undisturbed outward K^+ current). Membrane hyperpolarization, in turn, reduces glutamate release in the synaptic terminal. This constitutes the signal to the brain (for further image processing).

promoting the assembly of a fusion complex comprising the SNARE proteins synaptobrevin, SNAP-25 and syntaxin (Heidelberger et al., 1994; Morgans, 2000; Sudhof, 2013). With high light intensity, intracellular free Ca^{2+} reduces to nM levels, a concentration too low to bind all four binding sites on synaptotagmin and thereby arresting glutamate release. Depending on the synaptic connection, the released glutamate binds to either metabotropic receptors, which are expressed in bipolar-ON cells, or to ionotropic receptors, which are expressed in bipolar-OFF cells and on horizontal neurons. From here the signal is transmitted to the optic nerve (Figure 5-7).

ORDERS OF MAGNITUDE

It is estimated that a single photon, causing a single photoisomerization, can lead during the next second to the hydrolysis of more than 10^5 molecules of cGMP, leading to a closure of ~400 CNGA1 channels, so blocking the influx of as many as 10^7 Na^+ ions (and Ca^{2+}). The closed CNGA1 channels represent 3% of the total number that are open in the dark. The resultant hyperpolarization is ~1 mV and lasts (in rods) for ~1 s. This is sufficient to bring about a change in glutamate release at the synapse which impinges on the nerve cells that transmit the onward signal.

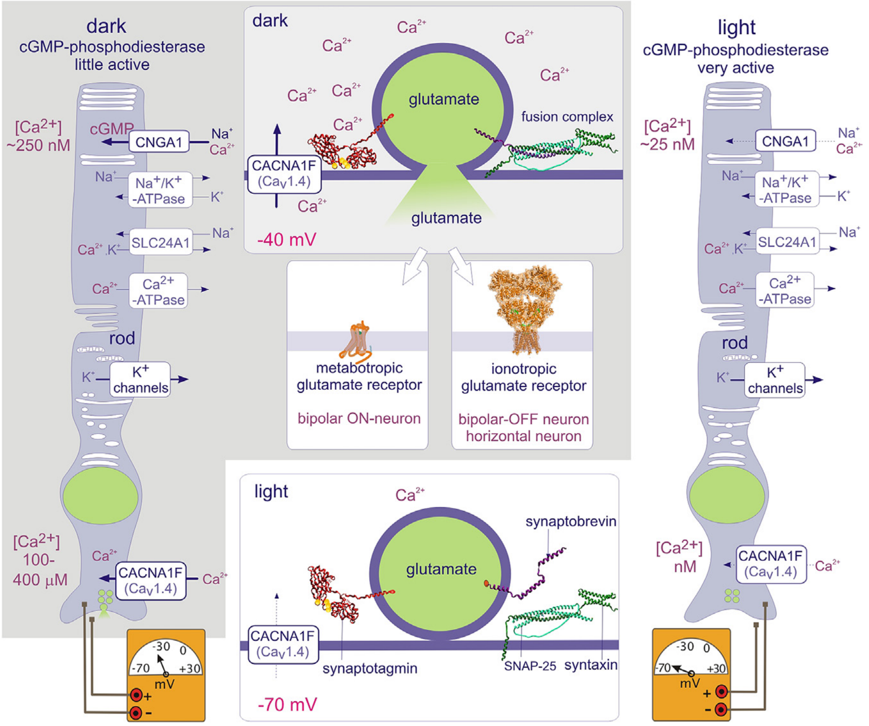


FIGURE 5-7 Linking membrane potential changes with synaptic glutamate release. Glutamate release is controlled by synaptotagmin. When bound to Ca^{2+}, it allows supercoiling of the SNARE proteins (the fusion complex) and hence fusion of secretion vesicles with the synaptic membrane. The light-induced hyperpolarization inhibits conductance of the voltage-sensitive Ca^{2+} channel (CACNA1F). The resulting dramatic reduction in intracellular Ca^{2+} in the synaptic terminal, descending from micro to nanomolar levels, prevents membrane fusion. Na^+, Ca^{2+}, and K^+ channel "leaks" are compensated by numerous transporters such as the Na^+/K^+ exchanger (SLC24A1), the Ca^{2+}- and the Na^+/K^+-ATPase.

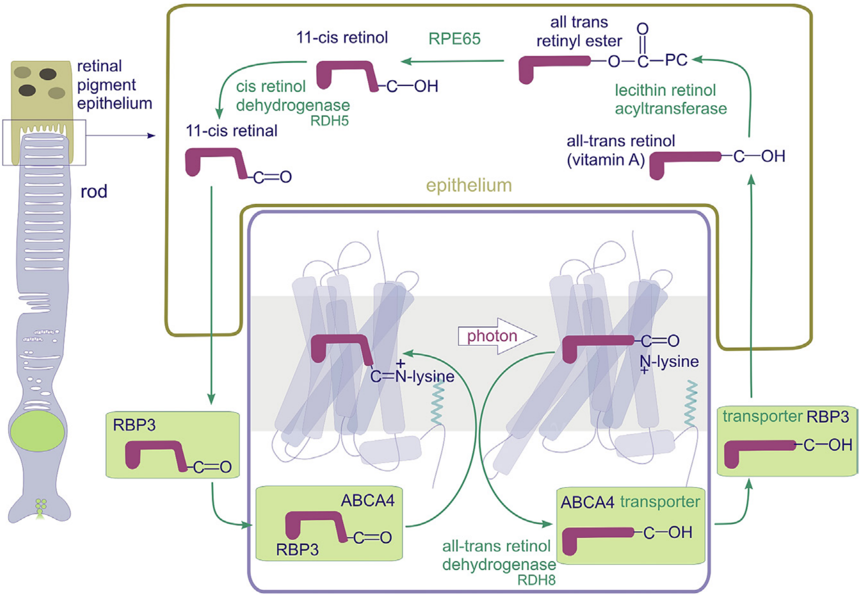


FIGURE 5-8 The visual cycle. The Schiff base of isomerized retinal is broken and the chromophore diffuses into the membrane. It is converted into all-*trans*-retinol, transported to retinal pigment epithelium where it returns to the *cis*-conformation. Upon conversion into 11-*cis*-retinal, the chromophore reenters the opsin and thus regenerates the visual pigment rhodopsin. Because of poor water solubility of retinoids, transport occurs through proteins (ABCA4 and RBP3).

The visual cycle; regeneration of the visual pigment rhodopsin

Upon photoisomerization, the Schiff base is broken and the chromophore is free to diffuse out of the binding pocket and into the plasma membrane. The free all-*trans*-retinal is converted into all-*trans*-retinol (by the dehydrogenase RDH8). Retinoids are only weakly water soluble and transport occurs with the help of proteins: ABCA4 within the rod cell and RBP3 for the transport to and from the retina pigment epithelium. Within the epithelium, all-*trans*-retinol is transferred to phosphatidylcholine by the enzyme lecithin retinol acyltransferase and then isomerized into 11-*cis*-retinol by RPE65. The product is next converted into 11-*cis*-retinal ready to be transported back to the rod cell. After reinsertion into the disc membrane, it somehow diffuses into the opsin-binding pocket and becomes covalently linked. The visual pigment rhodopsin is said to be regenerated (Figure 5-8) (Bridges and Alvarez, 1987; Lamb and Pugh, 2004).

Turning off the signal: the transducin GAP complex

The rate of decline of the photoresponse is primarily determined by the deactivation of transducin (Sagoo and Lagnado, 1997). The persistence of the active form of the α-subunit must depend entirely on the rate of hydrolysis of the bound GTP. However, the hydrolysis rate of isolated Gαt is far too slow, in the order of tens of seconds, to account for the physiological rate of recovery which is in the order of 1 s for rods (for cones the recovery is faster and occurs within 0.2 s). A complex of proteins acts as a transducin GTPase activator and these are RGS9, Gβ5, and RGS9BP. It lies in wait until the Gαt has established communication with PDE6γ, before it pounces (Figures 5-9).

Binding of PDG6γ enables PDE activity but it also facilitates the recruitment of RGS9 and this in turn causes hydrolysis of GTP (Angleson and Wensel, 1993; He et al., 1998). RGS9 belongs to the RGS-box family, group C, all of which are regulators of G-protein signaling and they promote hydrolysis of GTP (return to Chapter 2, Figure 2-31). RGS9 also carries a G-protein γ-like domain (GGL) with which it interacts with an atypical Gβ-subunit (Gβ5 or GBB5). The interface between GBB5 and the DEP-domain of RGS9 serves as a binding site for the third component, RGS9BP, which is a very short transmembrane protein that serves to recruit the complex to the membrane (see Figures 5-10 for molecular detail) (Slep et al., 2001; Cheever et al., 2008). Loss of any of the components of the transducin GAP complex slows down the termination of the photoresponse (Chen et al., 2000; Nishiguchi et al., 2004; Arshavsky and Wensel, 2013).

With the loss of the inverse agonist, metarhodopsin II could continue to propagate the light signal as a consequence of “spontaneous signaling.” It therefore is crucial to prevent its interaction with transducin, while waiting the covalent binding of a new 11-*cis*-retinal. This role is taken by the G-protein-regulated protein kinase GRK1. It too inserts into the binding pocket of the metarhodopsin II, although crystallographic data have not yet confirmed how, and phosphorylates the N-terminal sequence at numerous serine and threonine residues (S334, T336, S338,T340, T342, and S343 in human rhodopsin). These serve as a binding site for S-arrestin (SAG), thus blocking access of any effector. Binding of S-arrestin to the receptor, depending on the amino acids involved, in turn interferes with the regeneration of visual pigment rhodopsin (Sommer et al., 2012). We assume that the arrival of 11-*cis*-retinal brings about a conformational change that disables the interaction with GRK1. Subsequent dephosphorylation of rhodopsin by a serine/threonine phosphatase will reset the system, ready for a new phototransduction cycle.

The deactivation of PDE6 causes a rapid increase in cGMP followed by reopening of the CNAG1 channel and depolarization of the plasma membrane. The return to −40 mV reestablishes glutamate release at the synaptic terminal

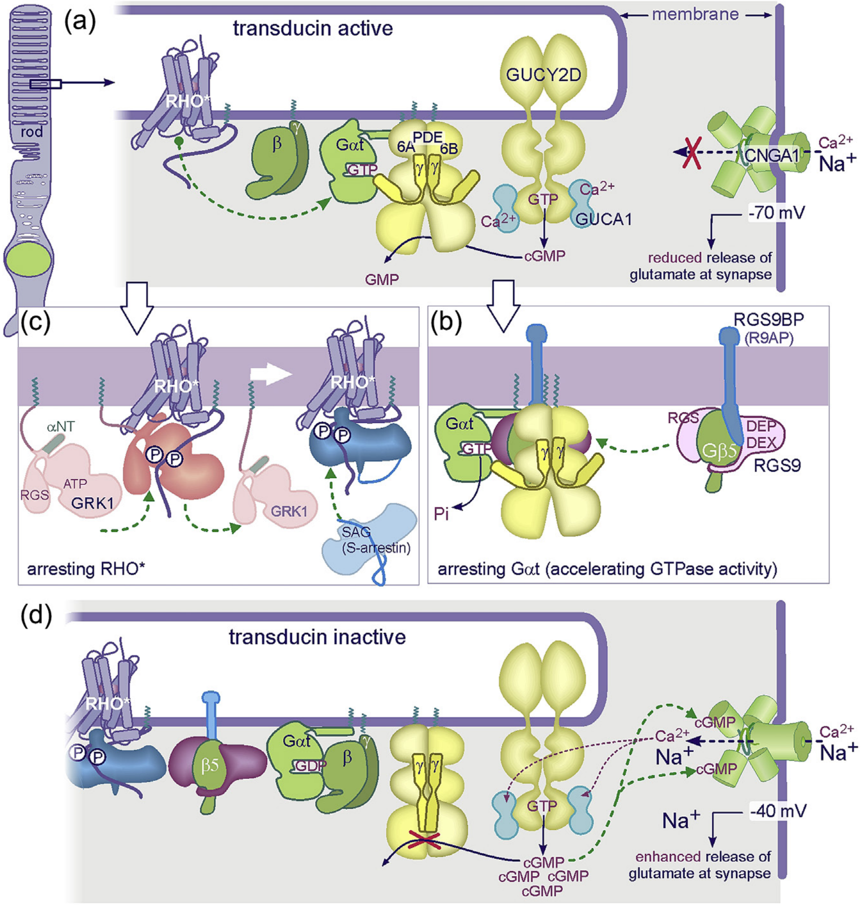


FIGURE 5-9 Turning off the photoresponse. (a) The starting situation comprises a photoexcited rhodopsin (metarhodopsin II) which activates Gαt leading to the activation of the PDE6A/B, the subsequent loss of cGMP, and the closure of the CNGA1 channel. The ensuing increase in membrane potential, descending to −70 mV, prevents neurotransmitter release. (b) The binding of inhibitory subunit PDE6γ to Gαt.GTP facilitates the recruitment of RGS9. This regulator of GTP-signaling is associated with the membrane through an interaction with Gβ5 and the membrane-anchor protein RGS9BP. Together, PDE6γ and RGS9, they accelerate GTPase activity and return Gαt into its inactive state. (c) In order to prevent reactivation of Gαt, GRK1 binds RHO and phosphorylates its N-terminal tail. This event is followed by the attachment of S-arrestin. The cycle can start again when RHO is regenerated through the insertion of a new 11-*cis*-retinal. (d) Loss of PDE6γ activity and an increase in GUCY2D activity (following a reduction in Ca^{2+}) increases the cGMP level, the CNGA1 channel opens, Na^+ and some Ca^{2+} enter, the membrane depolarizes, and glutamate release returns to "dark" levels.

(Figures 5-9(d)). Cyclic GMP is produced by guanylyl cyclase (GUCY2D), an enzyme controlled by Ca^{2+} in rod and cone cells. The control occurs through GUCA1 (GCAP1), which is a small protein composed of four EF-hands (Ca^{2+}-binding motif) and a fatty acid chain (N-terminal glycine-myristoylation).

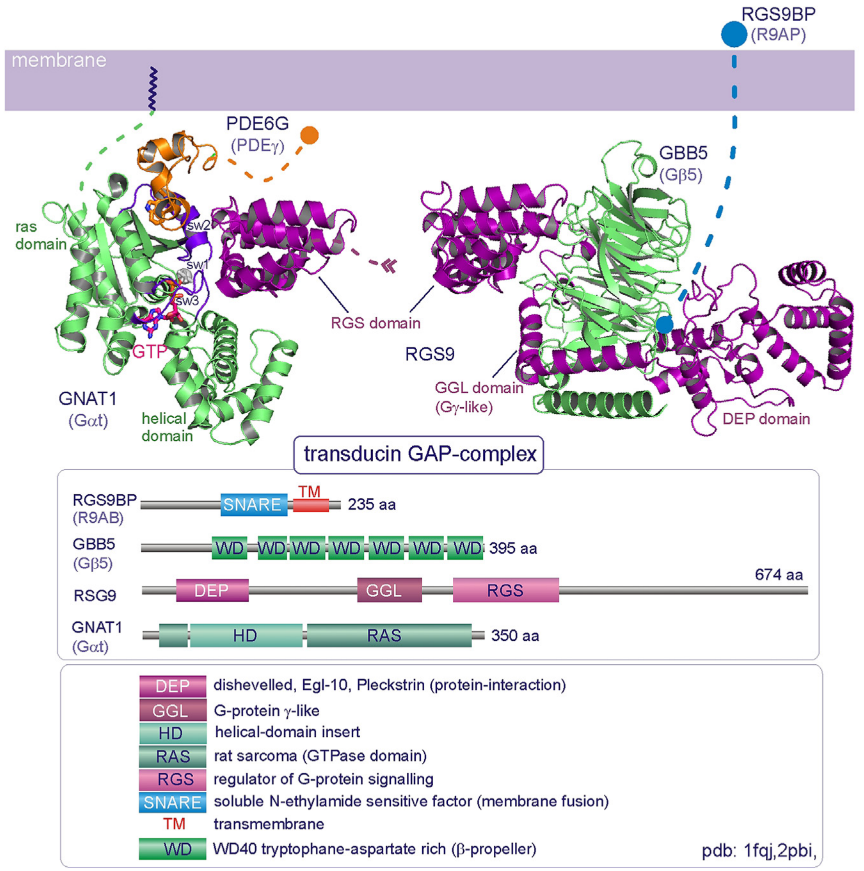


FIGURE 5-10 Molecular detail and domain architecture of the transducin GAP complex.

The fatty acid chain is constitutively buried inside the protein and does not play a role in membrane localization (Lim et al., 2013; Stephen et al., 2007; Baehr and Palszewski, 2009). GUCA1 is bound to the guanylyl cyclase and in the presence of high levels of intracellular free Ca^{2+} (>250nM) it inhibits activity, whereas it functions as an activator at low levels (30nM). What this means is that when the GNAG1 channels close, due to a lack of cGMP, the guanylyl cyclase will be activated and work at a higher regime, therefore rapidly reestablishing a μM concentration of the cyclic nucleotide.

ELECTRIC ACTIVITY OF ROD CELLS

Understanding of the first steps of the visual transduction mechanism has followed from the investigation of the interaction of light with single photoreceptor cells. To detect this, it is necessary to illuminate photoreceptors with very low light intensities so as to establish the minimal conditions for excitation. Using a whole-cell patch technique, changes in channel activities that we have described previously can be recorded as changes in current (photocurrent) in single rod outer segments. Current is detected when the CNAG1 channels close because this leaves a (transient) net outflow of positive ions (K^+) (Figure 5-11). Both the current and the duration of the pulse increase with the intensity of the light, meaning that

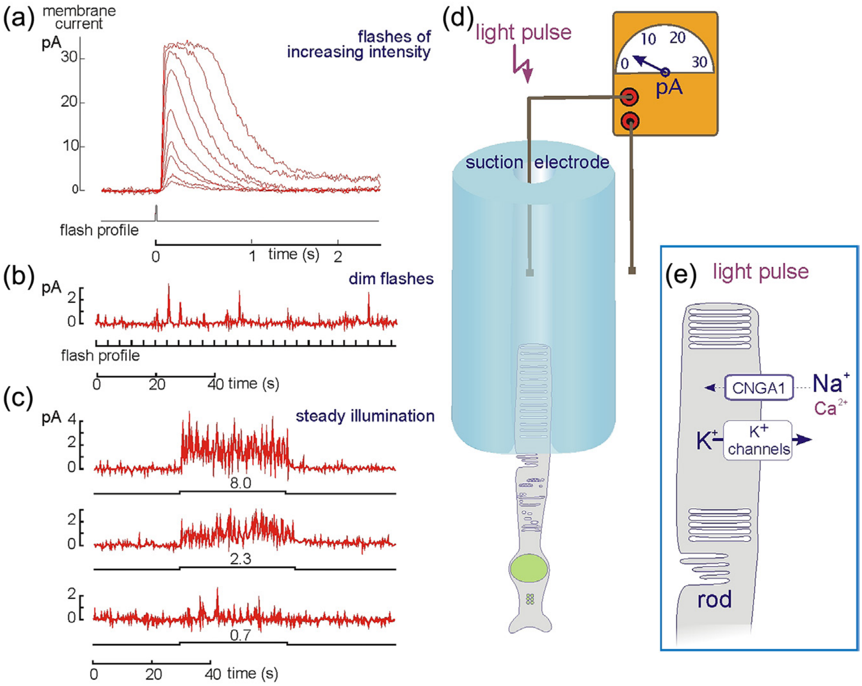


FIGURE 5-11 Detecting light responses in single rod cells. (a) Responses of a single retinal rod cell to flashes of light. Light flashes evoke transient photocurrents in the membrane of a single rod cell outer segment. The amplitude of the transient outward currents increases with flash intensity up to a saturating level of 34 pA; (wavelength: 500 nm, flash duration: 11 ms, photon density: 1.7–503 photons/μm^2, species: monkey, *Macaca fascicularis*). (b) Quantal responses of single retinal rod cells to dim flashes. The membrane current responses of a single rod cell to a train of dim flashes are variable in amplitude. This variability together with the presence of background noise gives the appearance of a sequence of successes and failures. Amplitude histograms (not shown) reveal a quantal response to light. The unitary events have an average amplitude of 0.7 pA and they correspond to the detection of a single photon; (photon density: 0.6 photons/μm^2, other conditions similar to panel (a)). (c) Response of a single rod cell to steady illumination. Records of photocurrents evoked by periods of steady illumination correspond to the superposition of random photon responses. The photon flux density is indicated below each trace (photons/μm^2 s). *(Adapted with permission from Baylor et al. (1984).)* (d) Drawing of the experimental setup. A rod cell or just the outer segment is hold in a suction peptide with electrodes at the inside and outside. Currents are recorded after applying light pulses. (e) A light pulse blocks the inward Na^+ and Ca^{2+} current giving rise to a (transient) net outward K^+ current (in the order of picoampères or pA).

an increasing number of CNAG1 channels are shut when photon intensity increases. However, the response saturates at a level of 34 pA (further increase in photon intensity provokes the same response). When applying a repetition of dim flashes, we observe a series of successes and failures; roughly 7 out of 10 flashes are effective. Moreover, these flashes reveal a quantal response to light. The unitary events have average amplitudes of 0.7 pA and they correspond to the detection of a single photon. When applying a steady illumination, the corresponding photocurrent corresponds to the superposition of single photon responses (giving rise to a repetition of a single or a multitude of 0.7 pA amplitudes).

Web resource

A rich resource about vision, Webvison, from the University of UTAH http://webvision.med.utah.edu/book/part-ii-anatomy-and-physiology-of-the-retina/photoreceptors/

SENSITIVITY OF PHOTORECEPTORS AND ADAPTATION TO CHANGING LIGHT INTENSITIES

With little knowledge of the basic physiology of the human eye, Hecht, Schlaer, and Pirenne in 1942 set about measuring its quantum sensitivity (Hecht et al., 1942). Using dark-adapted human subjects responding verbally to precisely calibrated single flashes (1 ms duration at 510 nm, close to the optimal wavelength for vision in dim light), they determined that the eye's detection limit corresponds to energies, incident at the cornea, in the range $2–6 \times 10^{-17}$ J, equivalent to 54–148 photons. To calculate the energy actually absorbed by the retinal receptors, they applied corrections to take account of losses due to reflection (4% at the cornea) and absorption by the ocular medium (50%). Also at least 80% of the light falling on the retina passes through unabsorbed. After all this, they estimated that number of photons absorbed by the visual pigment was in the range 5–14. Since this is very small in comparison with the number of retinal photoreceptors in the field illuminated, it was concluded that they are of such sensitivity that the coincidence of single photons impinging simultaneously on five cells is sufficient to strike consciousness in a human being (Hecht et al., 1942).

> The fact that for the absolute visual threshold, the number of quanta is small makes one realise the limitation set on vision by the quantum structure of light. Obviously the amount of energy required to stimulate any eye must be large enough to supply at least one quantum to the photosensitive material. No eye need be so sensitive as this. But it is a tribute to the excellence of natural selection that our own eye comes so remarkably close to the lowest level. ***Hecht et al. (1942)*****.**

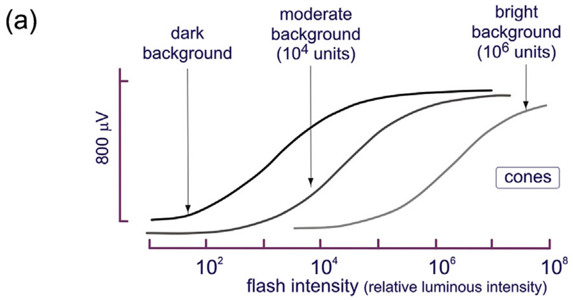
Much later, estimation of the quantum sensitivity of individual photoreceptors was performed electrophysiologically using suction electrodes applied to single rod cells obtained from the toad (Baylor et al., 1979). This approach avoids the subjectivity of human psychophysical experimentation and many of the assumptions made concerning the proportion of the light signal that actually reaches the photoreceptors. An outward membrane current was recorded during illumination and when dim flashes of light were applied, the current fluctuated in a quantal manner. Similar quantal responses have been observed in photocurrent records from primate rod cells; examples are shown in Figure 5-11. Each unitary event is considered to be the result of an interaction between a single photon and a single pigment molecule (rhodopsin).

Additionally, the human eye can detect transient events and recover rapidly. For example, when exposed to a train of dim flashes, we can distinguish, with our cones, individual events up to a frequency of 24 Hz. The flickering image is an enduring (and endearing) feature of old movies, in which the frame rate is slower than the flicker-fusion frequency of the audience. The faster frame rate of modern cinematography (and television) is just sufficient to allow successive images to fuse and give the impression of continuous motion. The flicker-fusion frequency of other species, such as some insects, can exceed 100 Hz.

> And when a Coal of Fire moved nimbly in the circumference of a Circle, makes the whole circumference appear like a Circle of Fire; is it not because the Motions excited in the bottom of the Eye by the Rays of Light are of a lasting nature, and continue till the Coal of Fire in going round returns to its former place? And considering the lastingness of the Motions excited in the bottom of the eye by Light, are they not of a vibrating nature? ***Sir Isaac Newton, Opticks.***

Although the human eye is able to sense fluxes of just a few photons per second, it can also detect subtle intensity differences under conditions of very bright illumination. This gives it a remarkable dynamic range (Figure 5-12).

For example, this response allows us to perceive light–dark contrasts at levels of illumination that range from that of a dark overcast night sky (about 10^{-6} cd/m^2) to the brilliance of sunlight reflected from snow fields (about 10^7 cd/m^2). The candela (cd) is an SI unit of measure of the intensity of visible light, called the luminous intensity. One candela is the luminous intensity, in a given direction, of a source that emits monochromatic radiation of frequency 540×10^{12} Hz (green light) and that has a radiant intensity in that direction of 1/683 W per steradian.



(b) dark adaptation (rendering the system sensitive, preventing an excess of cGMP)

- high levels of Ca^{2+}
  - recoverin (RCVRN) ⟶ inhibition of rhodopsin kinase (GRK1)
  - calmodulin (CALM) ⟶ inhibition of CNGA1 (Na^+, Ca^{2+}-channel)
  - GCAP1 (GUCA1A) ⟶ inhibition of GUCY2D (guanylyl cyclase)

light adaptation (rendering the system less sensitive, preventing depletion of cGMP)

- translocation of transducin to inner segment (reducing phosphodiesterase activation)
- low levels of Ca^{2+}
  - relief of inhibitory control of GUCY2D
  - relief of inhibitory control of GNGA1

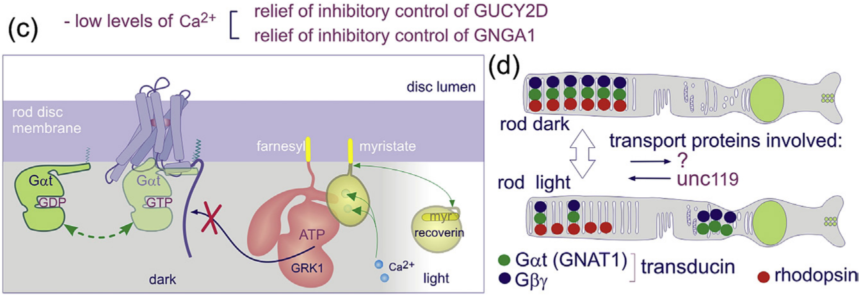


FIGURE 5-12 Dark and light adaptation. (a) The shift in the flash response curve of mammalian cone photoreceptors as the background light level increases. Cone responses (instantaneous illumination plus background) measured with a bipolar electrode. (b) dark and light adaptation strategies. (c) high levels of Ca^{2+} causes membrane association of recoverin and subsequent loss of GRK1. (d) long term adapation constitutes the removal of signalling components from the outer segment. *Modified from Valeton and van Norren (1983).*

Given that the incidence of a single photon can lead to the closure of 3% of the CNGA1 channels, it might seem that steady illumination of even modest intensity would cause closure of the whole lot. Indeed, if the response of the system was directly proportional to the *number* of photoreceptor molecules activated, then full saturation would be achieved at very low light levels. Yet, the human eye is capable of sensing small differences in the intensity of light against high background levels. Clearly, there must be an adaptive mechanism that reduces the amplification, so that there are always some open channels remaining even when the background is very bright. Overall the eye is responsive over an intensity range of about 11 orders of magnitude, a range that matches the extremes of the intensity of illumination at the Earth's surface during the normal day–night cycle (Rodieck, 1998). Much of this range can be accounted for by the ability of individual photoreceptors to adapt by decreasing their sensitivity. Thus, rod cells can adapt over two orders of magnitude and cones can adapt even more (over seven to nine orders, see Figure 5-12).

An important component of the adaptive mechanism by which the sensitivity is adjusted against ambient light levels is contributed by the cytosol concentration of free Ca^{2+}. It is essential to understand that we detect light when it brings about changes in the membrane potential. In the dark, high levels of intracellular free Ca^{2+} prevail and as we have already mentioned this inhibits guanylyl cyclase thereby preventing that cGMP will rise too high and make the system insensitive to low levels of activation of PDE. High levels of Ca^{2+}, through binding to calmodulin, also inhibit conductance of the CNAG1 channel. Lastly, it inhibits rhodopsin kinase (GRK1) so that very few active receptors have more time to interact with transducin. Inhibition of GRK1 occurs through binding to recoverin (RCVRN). Recoverin carries a myristate that, in the presence of Ca^{2+} is ejected from the protein and buried into the membrane. This allows the protein to bind to GRK1 and prevent access of the C-terminal tail of rhodopsin to the catalytic site (Komolow et al., 2009; Figure 5-12(c)).

With increasing light conditions, when PDE activity is elevated, the cation channels close in response to the hydrolysis of cyclic GMP and the concentration of Ca^{2+} declines from its dark level of about 300 to 30 nmol/l under strong illumination. This, in turn, accelerates the conversion of GTP to cyclic GMP due to the loss of Ca^{2+}/GCAP1-mediated inhibition and cGMP levels are restored. In the long term, as in the diurnal cycle, adaptation to intense illumination is also mediated by a redistribution of transducin between the outer and the inner segments of the retinal rod cells. As much as 90% of the transducin present in the outer segments in dark-adapted conditions can translocate into the inner segment of the light receptor on a timescale of tens of minutes (Sokolov et al., 2002; Zhang et al., 2011). This is accompanied by a corresponding 10-fold reduction in amplification of the signal, allowing the eye to maintain its sensitivity to changes in light against the brighter daytime background.

NOTE ON PHOTOTRANSDUCTION IN INVERTEBRATES

Phototransduction in *Drosophila*

We have described the basic elements of the signal transduction process as it occurs in the ciliary photoreceptors (rods and cones) of vertebrate eyes. The situation in invertebrates is very different. Where studied, the transduction cascade of ciliary photoreceptors leads to metabolism of cyclic GMP (cGMP) and modulation of cyclic nucleotide-gated (CNG) ion channels, while microvillar photoreceptors, which occur in the eyes of spineless creatures, use the more widespread phosphoinositide cascade, characterized by signaling through Gαq coupled to the effector enzyme phospholipase Cβ (PLCβ) and increasing conductance of nonselective cation channels belonging to the transient receptor potential (TRP) family. Apart from signaling, an important difference also concerns the regeneration of visual pigment. In *Drosophila*, the chromophore is 11-*cis*-3-hydroxy-retinal (and not 11-*cis*-retinal) and it has a very short visual cycle. After photoisomerization, the all-*trans*-3-hydroxy-retinal remains firmly attached and the activated metarhodopsin is so stable that it would have a half-life of more than 5h (Feiler et al., 1992). This would not be much help to flies but for the fact that the binding of arrestin (arr) sensitizes the system to a second photon, this one of longer wavelength (580nm, orange). This triggers the reinstatement of 11-*cis*-3-hydroxy-retinal. Finally, we note that whereas vertebrate eyes have solved the problems of absolute sensitivity, speed of response and dynamic range by using two classes of photoreceptors: rods and cones, invertebrate photoreceptors express only one type of photoreceptor. These photoreceptors not only detect single photons, they often do so more rapidly than rods and yet can still adapt to signal in full sunlight, when photon fluxes per photoreceptor can exceed 10^6 effectively absorbed photons per second (Fain et al., 2010; Hardie, 2012).

Vertebrates also possess microvillar photoreceptors which express rhabdomeric opsins, coupled to Gαq and PLC. These photoreceptors do not occur in retina but are found in retinal ganglion cells and their function is to mediate papillary contraction and circadian entrainment (Shichida and Masuyama, 2009).

The *Drosophila* compound eye is composed of ~800 ommatidia, each with a facet lens and eight photoreceptor cells, and a number of pigment and other accessory cells (Figure 5-13). Each photoreceptor has a light-absorbing rhabdomere, a rod-like structure 80–100μm long and 1–2μm in diameter, composed of roughly 30,000 tightly packed microvilli. Microvilli, in contrast to cilia which require physical support from microtubuli, are supported by actin filaments. In cross section, the ommatidium forms a hexagonal array of photoreceptor cells with six peripheral rhabdomeres (R1–R6) containing the same rhodopsin (ninaE, λmax = 480nm), and a central rhabdomere formed by R7 and R8 (placed proximal and distal respectively). R7 cells detect near UV light with a λmax of 340nm, whereas R8 expresses a variety of visual pigments with a λmax of 370, 440, and 510nm. The R8 cells subserve color vision. All major components of the transduction cascade from rhodopsin

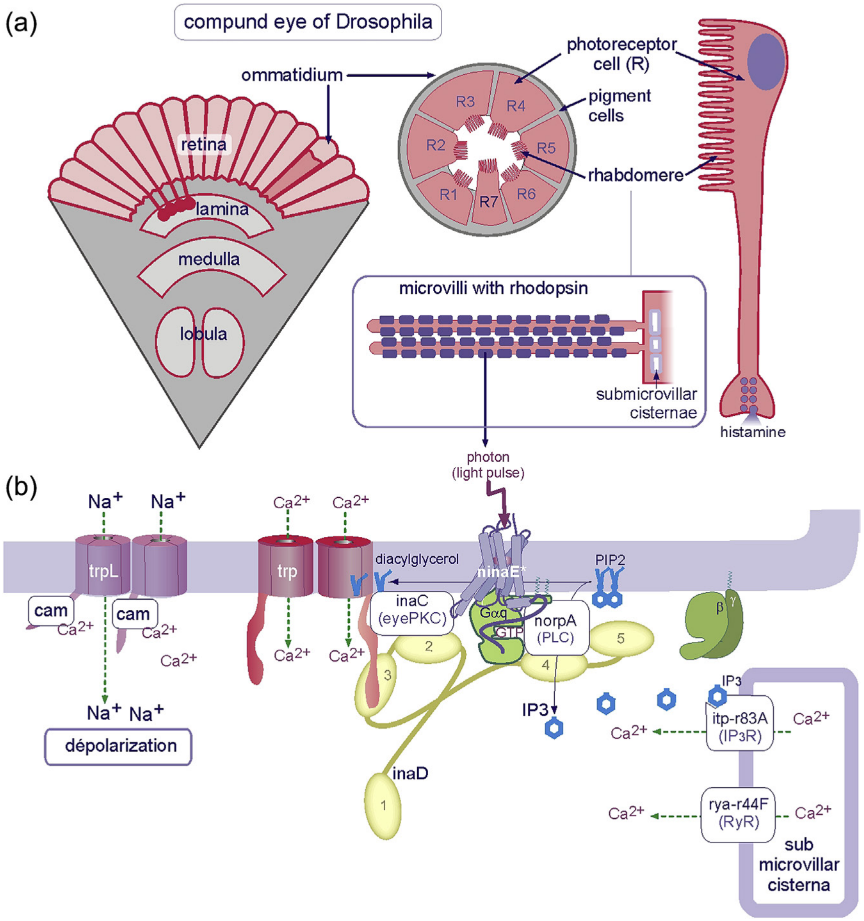


FIGURE 5-13 Phototransduction in *Drosophila*. (a) Details of the compound eye. (b) Activation of the cascade is initiated by a photon that causes the conversion of 11-hydroxy-*cis*-retinal to the all-*trans* form. This causes the binding and activation of Gαq, which activates norpA (equivalent of PLCβ) and results in the production of membrane-embedded diacyglycerol and cytosolic inositol-1,4,5-trisphosphate (from PIP$_2$). As a consequence, through an as yet poorly understood mechanism, both trpL and trp channels open, giving rise to a net inward current of Na$^+$ and Ca^{2+}. The membrane potential drops and together with an increase in Ca^{2+} this is coupled to an increase in the release of histamine at the synaptic terminal in the lamina area.

to the cation channels are localized in the microvilli, and the immediate effect of absorption of a photon is likely confined to the microvillus where it was absorbed. This ultra-compartmentalization promotes rapid response by minimizing diffusional delays and increasing the effective concentration of reactants (Hardie, 2012).

A typical microvillus has a membrane area of ~0.2 mm^2 comprising ~280,000 phospholipids of which 1–2% are PIP$_2$. Each microvillus contains about 1000 rhodopsins, 50 G-proteins, 100 PLCβ's, 25 TRP, and 2 TRPL channels. As part of the INAD signaling complex, PLCβ and TRP channels are likely to be effectively immobile, as is rhodopsin. Therefore, G-proteins presumably act as diffusible shuttles that transfer the signal from active rhodopsin to PLCβ (Hardie, 2012).

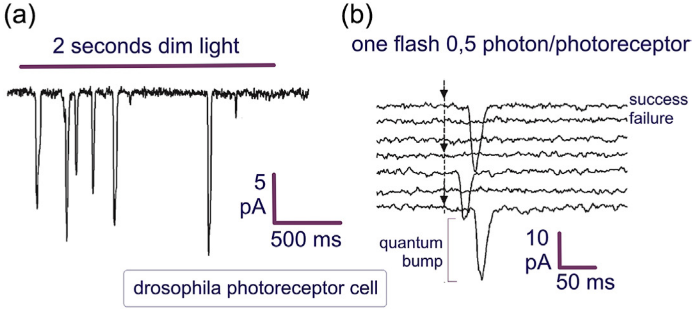


FIGURE 5-14 Detecting light responses in a single photoreceptor cell of *Drosophila*. (a) Exposing the photoreceptor cell to dim light provokes repeated inward currents of 25 pA (b) The quantal response is illustrated here with the application of a very dim light flash. Photoreceptor cells either respond with a single quantum pump (10 pA and lasting for 40 ms) or they fail to do so.

In some ways, phototransduction in flies appears to follow more conventional pathways. Here, the photoexcited rhodopsin is coupled through the G-protein Gαq (Galpha49B), not transducin. This regulates phospholipase Cβ (norpA), producing inositol triphosphate (IP$_3$) and diacylglycerol, and results in the elevation of intracellular Ca^{2+} and the activation of protein kinase C (inaC). As with vertebrates, these events initiate both electrical and chemical signals, but here the consequence is the opening, not the closure, of plasma membrane ion channels of the transient potential family (trp and trp-like). How the signaling events linking activation of norpA with the opening of the channels is not clear yet. Whatever the cause, opening of the channels causes a very transient depolarization due to an inward Na$^+$ (trpL) and Ca^{2+} current (trp) (Figure 5-14). At the resting potential (–70 mV), responses to single photons ("quantum bumps") have a mean amplitude of 10 pA representing the opening of 15 channels (compared to hundreds needed for a signal in human rod). Quantum bumps are brief in duration (20 ms half-width) and generated with a variable latency (15–100 ms). The macroscopic response to brief flashes (impulse response) containing up to at least several 100 photons is accurately predicted by the linear superposition of the underlying bumps (Henderson et al., 2000). The transient depolarization leads to the release of histamine (possibly by means of voltage-sensitive Ca^{2+} channels) at the synaptic terminals in the lamina area of the compound eye. From here the signal is integrated in the medulla and then sent to the lobula area.

Signal termination

The increase in intracellular free Ca^{2+} plays several roles in ensuring the deactivation of the photosignal. It inhibits norpA (PLCβ) and through binding to calmodulin it inhibits the trp-like channel. Together with diacylglycerol, Ca^{2+} activates the serine/threonine protein kinase inaC (PKC) which phosphorylates and inhibits trp (Cook et al., 2000; Huber et al., 1996; Liu et al., 2000). NorpA, inaC, and trp are held together by a scaffolding protein, inaD. Its role is to maintain the signaling components in high concentrations in the rhabdomere and in stoichiometric relationship (Tsunoda et al., 1997). Loss of inaD prolongs the depolarization phase. The photoexcited rhodopsin, active metarhodopsin, would normally continue to excite the phototransduction cascade, where it not that access to Gαq is rapidly blocked by binding of arrestin (arr). *Drosophila* expresses two isoforms, arr1 and arr2, of which arr2 is the most important. Contrary to the knockout of arr1, loss of arr2 causes a pronounced deactivation defect, with responses decaying slowly over 1–2 s (rather than 100 ms on average) (Dolph et al., 1993).

Many of the underlying genes were identified by random mutagenesis screens in the 1960s and using electroretinograms to detect anomalies (Pak et al., 1969; Hotta and Benzer, 1969). We show only a fraction of the 30 genes or so that were shown to act directly or indirectly in the phototransduction pathway.

> Is not Vision preform'd chiefly by the Vibrations of this medium, excited in the bottom of the Eye by the Rays of Light, and propagated through the solid, pellucid and uniform Capillamenta of the optick Nerves into the place of Sensation? And is not Hearing perform'd by the Vibrations either of this or some other Medium, excited in the auditory Nerves by the Tremors of the Air, and propagated through the solid, pellucid and uniform Capillamenta of those Nerves into the place of Sensation? And so of the other Senses. ***Sir Isaac Newton, Opticks.***

OLFACTION

CHEMOSENSORS

Olfaction occurs in the olfactory epithelium which is part of a broader chemoreception system distributed over several specialized organs. Insects are particularly well equipped, they have chemoreceptive antennae, maxillary palps, and sensors on their legs, wings, genitalia, and on the proboscis. Rodents have an olfactory epithelium, a vomeronasal organ, and taste buds. In humans we count two organs, the olfactory epithelium and numerous taste buds. We distinguish receptors for odorants, tastants, pheromones, and pathogens (Figure 5-15). The human genome contains a large number of genes coding for chemoreceptors of which, however, a large majority are nonfunctional (pseudogenes). Thus, humans have lost nearly all pheromone receptors (TAAR, V1R, V2R) and with them a functional vomeronasal organ. The few remaining pheromone receptors have been detected in the olfactory epithelium (Dulac and Torello, 2003). Of the 802 human olfactory receptor genes, only 388 are functional against 822 in dogs, 1152 in cows, and 1259 in rats (Nei et al., 2008). Nearly all vertebrate chemosensors are metabotropic receptors belonging to the superfamily of GPCRs or seven transmembrane-spanning proteins. They are coupled to various signaling pathways, involving adenylyl cyclase, guanylyl cyclase, or phospholipase C (Zufall and Munger, 2001). The sour taste receptor makes the exception; there is convincing proof that it is an ion channel belonging to the TRP family (Chandrashekar et al., 2006). Arthropods, such as *Drosophila*, also employ seven transmembrane-spanning proteins but, beware, their topology is different and they operate in an ionotropic fashion, acting as multimeric ligand-gated ion channels. Moreover, the antennae carry glutamate receptor-like proteins (Silbering and Benton, 2010; Benton, 2009).

Humans express 388 functional olfactory receptors (and 414 pseudogenes), 6 functional TAAR (and 3 pseudogenes), 5 functional V1R (and 115 pseudogenes), 0 functional V2R (and 20 pseudogenes), 3 functional T1R (and 0 pseudogenes), and 25 functional T2R (and 11 pseudogenes). The olfactory genes can be subdivided into α, β, γ, δ, ε, ζ, η, θ of which only α (class I) and γ (class II) are expressed in humans (detecting airborne odorants) (Nei et al., 2008).

Web resources

- The HUGO Gene Nomenclature Committee (HGNC), http://www.genenames.org/genefamilies/OR
- The human olfactory data explorer (HORDE), Weizmann Institute of Science http://genome.weizmann.ac.il/horde/
- The olfactory receptor database (ORDB) from the SenseLab, Yale University, https://senselab.med.yale.edu/ORDB/

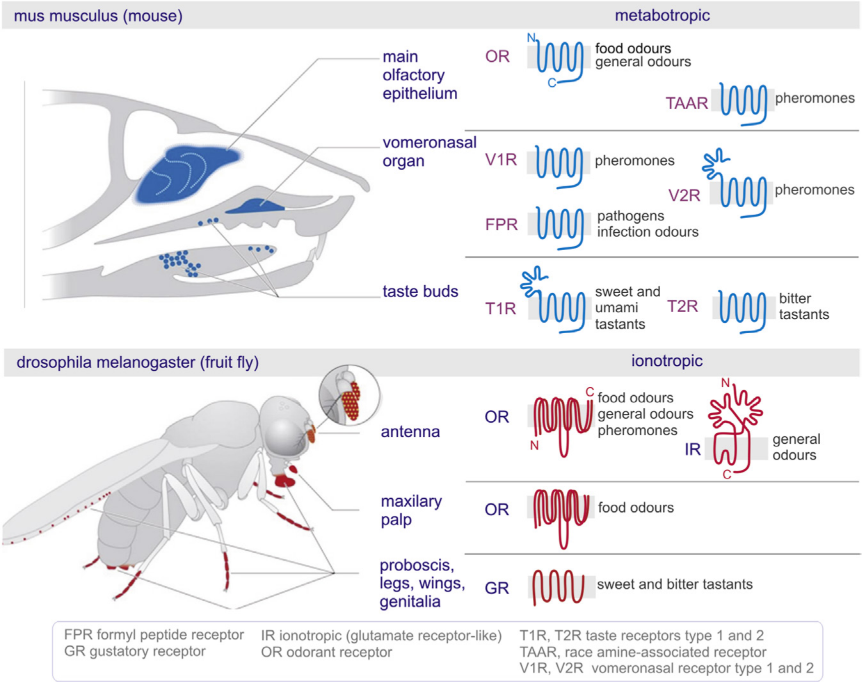


FIGURE 5-15 Chemosensory organs, chemosensors (receptors), and their putative ligands in the mouse and the fruit fly. Vertebrates almost exclusively use metabotropic receptors which couple to G-proteins and control ion channels via the production of second messengers, whereas insects (and possibly all arthropods) predominantly use ionotropic mechanisms, where the receptors are ligand-gated ion channels. Note the different topology between the seven transmembrane proteins in the mouse and the fruit fly. The gustatory receptors (GR) in *Drosophila* are still poorly characterized but their sequence homology with olfactory receptors (OR) make it plausible that they function as ion channels. Acid and salt receptors in vertebrates may also operate as ligand-gated ion channels (not shown). The OR in *Drosophila* occur as dimers with coreceptors hence the double line. *Image of the mouse head is adapted from Matsunami and Amrein (2003). Image of* Drosophila *from Silbering and Benton (2010). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.*

OLFACTORY EPITHELIUM

The human olfactory epithelium is situated in the nasal cavity. It consists of bipolar olfactory sensory neurons whose axons cross the cribriform bone (fenestrated part of the skull) and project into the olfactory bulb. Here, they connect with mitral cells in anatomical structures named glomeruli. Glutamate is the neurotransmitter connecting the sensory neuron with the mitral cells. There are numerous lateral contacts in the glomeruli allowing for ample integration of individual sensory neuron signals. The convergence is such that a single mitral cell may receive input from some 1000 olfactory sensory neurons. The axons of the mitral cells constitute the olfactory tract which conveys the signals to the primary olfactory cortex in the brain. Regardless of their location on the surface of the nasal epithelium, the olfactory sensory neurons bearing the same chemoreceptor are gathered together in the same glomerulus within the bulb (Mombaerts et al., 1996; Feinstein and Mombaerts, 2004). The dendrites of the sensory neurons terminate as ciliary extensions, supported by microtubules, 30–200 μm long and counting roughly 20 on each neuron. They bear the odorant receptors which project into the mucus layer lining the upper reaches of the nose. These dendritic terminations represent the single point at which cells of the central nervous system are exposed to the environment outside the body (Figures 5-16 and 5-17(a)).

It is said that St Jerome remarked of St Hilarion that he had the gift of knowing what sins and vices anyone was inclined to by smelling either the person or their garments. By the same faculty, he could discern good feelings and virtuous propensities (Room, 1999). Indeed, this may, or may not be true. We have no way of knowing. What is true is that dogs can distinguish between the smell of T-shirts worn by nonidentical but not by identical twins.

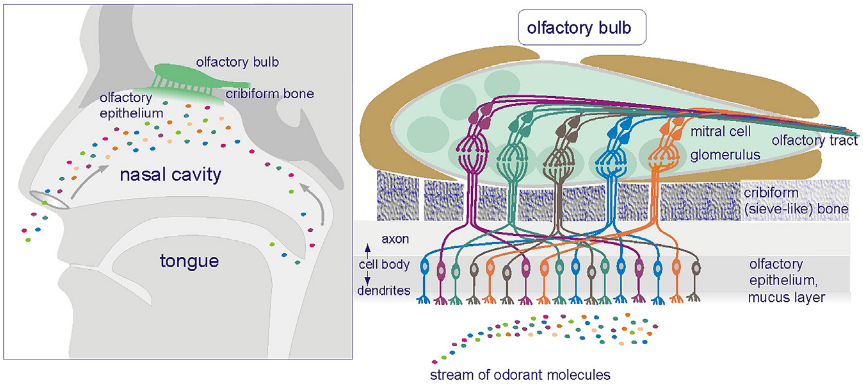


FIGURE 5-16 Anatomy of the human olfactory epithelium and olfactory bulb.

The human olfactory system is capable of sensing and distinguishing several hundred different molecules (odorants) and a seemingly limitless number of different smells (effectively, combinations of odorants). All of these compounds are volatile, none has a molecular weight much greater than 300 Da and all have the ability to partition between lipid and aqueous phases. Some of the odorants are liberated through mastication of food and are appreciated as "taste" rather than "smell." Smell plays an important role in tasting as witnessed by the loss of taste when we produce an excess of nasal mucus, which hinders access to the sensory neurons, in response to a viral infection of the upper respiratory tract (common cold). The human olfactory epithelium is formed from ~6 million sensory neurons (ORNs), whereas the rat has as many as ~50 million (and as a consequence is much more sensitive). The sensory neurons are surrounded by the supporting cells and the epithelium harbors numerous Bowman's glands that secrete mucus into the upper reaches of the nasal cavity.

Mitral: pertains to the form of a miter, a bishop's hat.

ODORANT RECEPTOR SIGNALING

Odorant receptors are seven transmembrane-spanning proteins (Buck and Axel, 1991) which are coupled to the olfactory G-protein, of which the Gα-subunit is named Gαolf or Gαo (Jones and Reed, 1989). The human genome name is GNAL (return to Chapter 2, Figure 2-29, for classification of G-proteins). Lacking the possibility of carrying out the ligand-binding experiments that have played such an important role in classical pharmacology, clues such as sequence variability among the receptors have been exploited to indicate the zones responsible for odorant binding. From this and from mutagenesis and modeling studies of the mouse eugenol receptor (mOR-EG), it has been revealed that the transmembrane helices 3, 5, and 6 harbor sites of odorant attachment. A set of 10 amino acids was identified, which, depending on the type of odorant, participate in binding with variable importance (Baud et al., 2011). There is no reason to think that the olfactory receptors possess the high specificity toward their ligands characteristic of most other receptors. In general, it appears that they bind multiple odorants of related structure and that most odorants are capable of activating several different receptors (Figure 5-17). Thus, different odorants activate different sets of glomeruli (Firestein, 2004).

Upon binding of the odorant, the receptor activates in a manner similar as described for rhodopsin (this chapter) and adrenaline (Chapter 3). This causes the exchange of GDP for GTP on Gαolf (Breer et al., 1990), followed

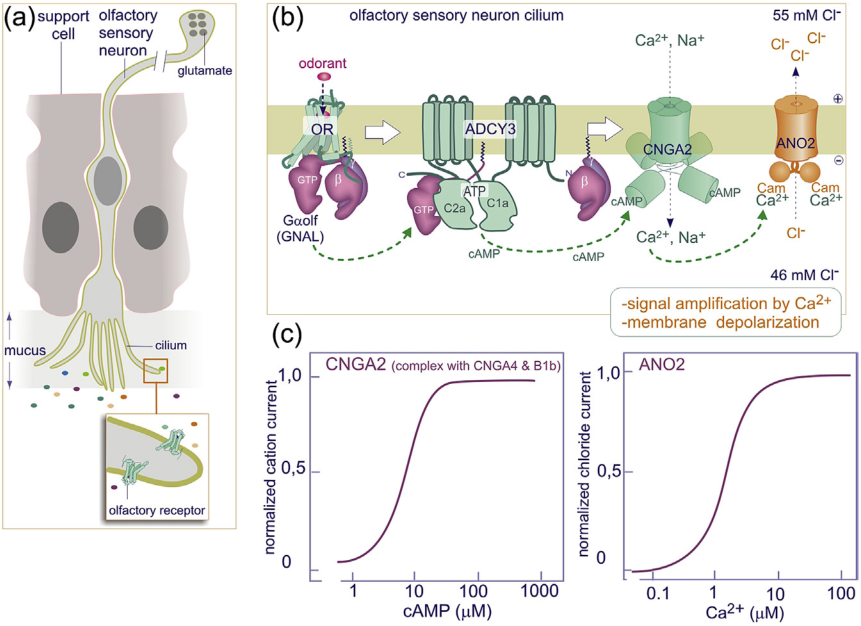


FIGURE 5-17 Olfactory signal processing. (a) Detail of the olfactory epithelium, a mixture of olfactory sensory and supporting cells. Cilia of the sensory neurons, exposed to the mucus layer, carry the olfactory receptors (each neuron one type of receptor). (b) The signaling cascade in an olfactory sensory neuron cilium starts with the activation of the olfactory G-protein. The Gαolf (GNAL) bound to GTP attaches to and activates adenylyl cyclase-3, resulting in the production of cAMP. This causes an increase in conductivity of the cyclic nucleotide-sensitive cation channel (a heterotetramer composed of CNGA2, A4, and B1b) leading to entry of Na^+ and Ca^{2+}. The increased cytosolic Ca^{2+} opens the ANO3 chloride channel and this causes the membrane depolarization that leads to the release of histamine at the synaptic terminal. (c) These two dose–response curves illustrate nicely the cascades of signal amplification. Raising the cAMP brings about the opening of the CNG channel and the ensuing rise in Ca^{2+} opens the Cl^- channels, which are so abundantly expressed that they bring about a strong membrane depolarization.

by a functional dissociation of the heterotrimeric G-protein complex and subsequent activation of adenylyl cyclase-3 (ADCY3) (for a description of adenylyl cyclase, return to Chapter 3, Figure 3-13) (Pace et al., 1985; Wong et al., 2000). To illustrate its importance, adenylyl cyclase-3 knockout mice cannot smell and are said to be anosmic (Wong et al., 2000). The cAMP formed interacts with a cyclic nucleotide (CNG) channel composed of four subunits: CNGA2 (twice), CNGA4, and CNG1b (Nakamura and Gold, 1987; Leinders-Zufall et al., 1997). All four subunits carry a cAMP-binding pocket. This causes an increase in conductivity for Na^+ and Ca^{2+}, the predominant cations in the mucus. The entry of Na^+ brings about a first reduction in membrane potential, whereas the entry of Ca^{2+} affects a second channel, ANO2, which is a Ca^{2+}/calmodulin-sensitive Cl^- channel

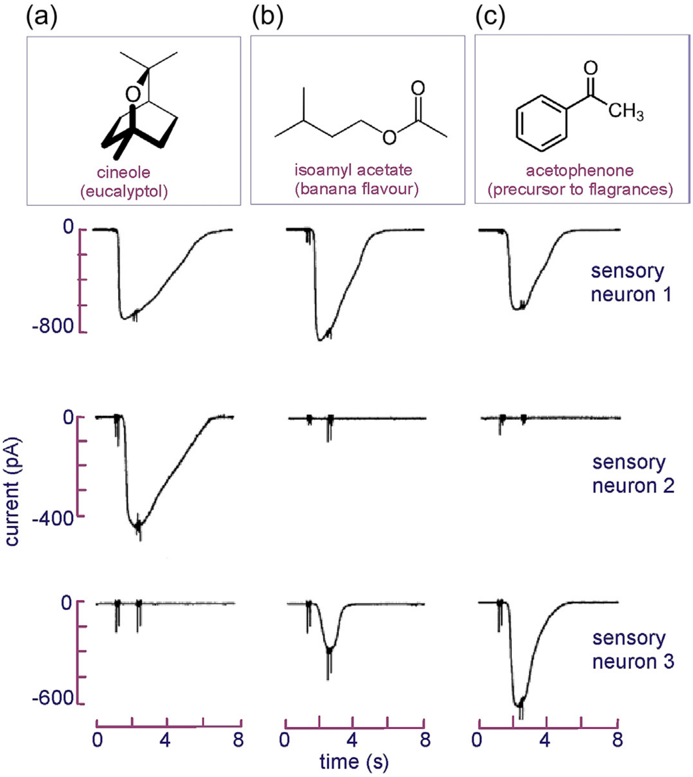


FIGURE 5-18 Current responses upon exposure of mechanically isolated sensory neurons, of the salamander *Ambystoma tigrinum*, to three different odorants (cineole, isoamylacetate, and acetophenone). Note the promiscuity of ligand binding, neuron 1 responds to 3 different odorants. *Image adapted with permission from Firestein et al. (1993).*

(see also Chapter 4, Figure 4-20). This causes further membrane depolarization for a period of one or more seconds (Figure 5-18). ANO stands for "anoctamin," thus named because it is anion selective and has eight (oct) transmembrane segments. Normally Cl^- levels are high outside (116 mM) and low inside the cell (20 mM) and channel opening would cause an inward (hyperpolarizing) current (Boron and Boulpaep, 2011). The situation is different in the olfactory epithelium. The cells exhibit unusual high Cl^- contents (around 46 mM), whereas the mucus has relatively low concentrations (around 55 mM). This, together with the prevalent membrane potential (negative on the inside relative to the outside), leads to an outward flow of chloride ions when ANO2 channels increase their conductivity. The chloride current is the major amplifying step in odorant detection (equivalent to the voltage-sensitive Na^+ channels in other excitable cells). ANO2 causes 80% of the current and is expressed in excess, about ninefold, of CNG channels (Takeuchi and Kurahashi, 2005;

Stephan et al., 2009). Membrane depolarization increases the release of glutamate at the synapse where sensory neurons connect with mitral cells in the olfactory bulb. The mitral cells express excitatory glutamate receptors, type NMDA and AMPA (O'Connor and Jacob, 2008) and their axons conduct the action potential into the brain.

The elevated intracellular level of chloride ions is maintained by the $Na^+/2Cl^-/K^+$ symporter (SLC12A2 or NKCC1) and by a Cl^-/HCO_3^- antiporter (SLC4A1) (Restrepo, 2005). As a consequence, chloride reversal potential in the olfactory sensory cells is near 0 mV so the response is depolarizing when the ANO2 channels open. It is suggested that olfactory sensory neurons employ the outward flow of Cl^-, rather than an inward flow of Na^+ to achieve membrane depolarization, as a fail-safe, in case the availability of Na^+ in the nasal mucus, a very uncontrollable environment, is insufficient to warrant a full depolarization. This depolarization cannot really be considered an "action potential" for the reason that it does not propagate. "Sensory potential" would be an appropriate term.

Recovery and adaptation

Olfactory signals last for 1 or 2 s. Calcium plays as much a role in terminating as it does in initiating the olfactory signal. It binds calmodulin attached to the CNG channel (present on subunits A2 and B1b) and this causes a 20-fold drop in the sensitivity to cAMP. This role of Ca^{2+} allows for adaptation. In the presence of high levels of odorants, all the channels would be open and changes would not be detectable. The ensuing increase in intracellular free Ca^{2+} renders CNG less sensitive to cAMP and higher doses are required for signal induction. At very high Ca^{2+} levels, the channel effectively closes (Figure 5-19; Song et al., 2008; Trudeau and Zagotta, 2002). ANO2 too is inhibited by high levels of Ca^{2+}, it is suggested that the protein has both stimulatory low-affinity and inhibitory high-affinity binding sites (Vocke et al., 2013).

Calcium ions also stimulate calcium calmodulin-dependent protein kinase-2 (CAMKK2), resulting in the phosphorylation and inactivation of ADCY3 (Wei et al., 1998). Together with a Ca^{2+}-mediated activation of PDE1C (Cygnar and Zhao, 2009), this results in a loss of cAMP. Finally, Ca^{2+} is removed from the olfactory cilium by the action of SLC24A4, a $Na^+/Ca^{2+}/K^+$ cotransporter protein (Stephan et al., 2011). Normally, Gαolf would be the target of regulators of G-protein signaling regulators (RGS proteins) but the studies directed to elucidate their role demonstrated an unexpected direct inhibition of adenylyl cyclase-III by RGS2 (Sinnarajah et al., 2001). Moreover, prolonged elevated levels of Ca^{2+} elevate RGS2 mRNA expression and, therefore, rather than rapid attenuation of the receptor signal, RGS2 may mediate long-term adaptation to odorants.

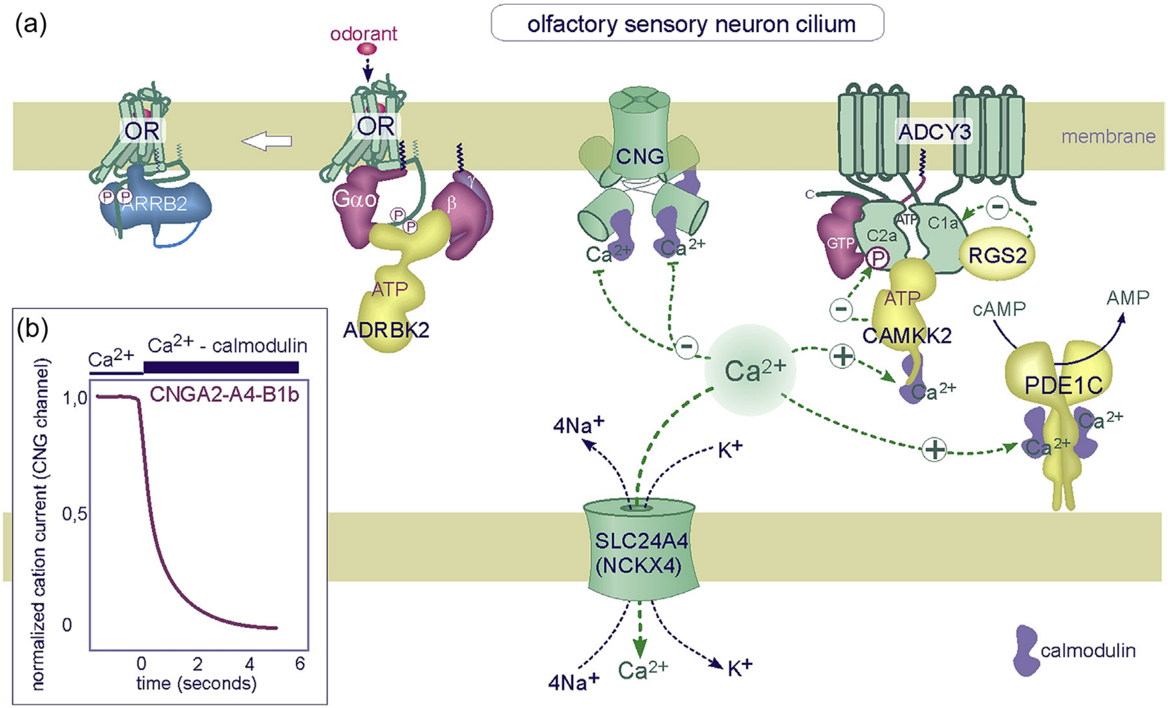


FIGURE 5-19 Recovery and adaptation of olfactory sensory neurons. (a) A multitude of events terminates the olfactory response: phosphorylation of the receptor by ADRBK2, binding of ARRB2 to the phosphorylated receptor, inhibition of conductance of CNG by Ca^{2+}/calmodulin, inhibition of ADCY3 by RGS2 and by Ca^{2+}/Calmodulin/CAMKK1 and by activation of PDE1C, which causes removal of cAMP. Ca^{2+} itself is removed through a Na^{+}/K^{+}/Ca^{2+} antiporter. (b) Graph illustrating the time-course of Ca^{2+}-mediated inhibition of the CNG channel.

The olfactory receptor neurons contain a highly specialized isoform of phosphodiesterase, PDE1C, having high affinity for Ca^{2+} (K_M 1.4 µmol/l). More than this, it has a higher affinity for cAMP than any other brain isoform (Yan et al., 2003). Thus, the binding of odorants to receptors not only initiates the cyclic AMP signal pathway, but also signals its termination.

We have dealt with the effectors, but the odorant receptors themselves are also inactivated in a fashion that is characteristic of all GPCRs. First, the C-terminal gets phosphorylated by the protein kinase ADRBK2 (Dawson et al., 1993). This abrogates attachment of Gαo. Finally, β-arrestin-2 (ARRB2) binds the phosphorylated receptor and effectively desensitizes the system (until a protein phosphatase resets the balance to zero). Odorant receptors are internalized (Mashukova et al., 2006). The relative contribution of any one mechanism to recovery and adaptation is unknown.

Web resource

KEGG, Kyoto encyclopedia of genomes and genes, pathway map 04740

Linda Buck and Richard Axel shared the Nobel Prize in Physiology and Medicine "for their discoveries of odorant receptors and the organization of the olfactory system."

As it happens, this was not the first time the olfactory system has Figured in a Nobel Prize. Nearly a century earlier, in 1906, this same Prize was awarded to Ramon y Cajal for his formulation of the neuron doctrine, which, as he pointed out in his Nobel lecture was based on work in the cerebellum, the spinal cord, the retina, and the olfactory bulb. For more information consult www.Nobelprize.org.

Recommended articles related to the discovery of olfactory receptors and how they connect with the brain:

- Buck, L., Axel, R., 1991. A Novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175–187.
- Buck, L.B., 2004. The search for odorant receptors. Cell 116, 117–119.
- Firestein, S., 2005. A Nobel nose: the 2004 Nobel Prize in Physiology and Medicine. Neuron 45, 333–338.
- Zou, D.J., Chesler, A., Firestein, S., 2009. How the olfactory bulb got its glomeruli: a just so story? Nat. Rev. Neurosci. 10, 611–618.

OTHER SIGNALING PATHWAYS INVOLVED IN CHEMOSENSING

Sensing of CO_2 through activation of guanylyl cyclase

A small subpopulation of mouse olfactory sensory neurons (≤0.1% of neurons in the main olfactory epithelium) lacks components of the cAMP-signaling pathway but instead expresses guanylyl cyclase-D (a transmembrane protein), the cGMP-selective CNGA3 channel (also expressed in cone photoreceptors) and PDE2, a cGMP-stimulated PDE. The axons of these sensory neurons project to a special set of glomeruli, referred to as the "necklace glomeruli" (Fülle et al., 1995; Juilfs et al., 1997; Zufall and Munger, 2001). These sensory neurons also express carbonic anhydrase II, an enzyme that converts CO_2 into HCO_3^- and H^+. It has been shown that the guanylyl cyclase-D is activated by $HCO3^-$. The ensuing production of cGMP increases conductivity of CNGA3, resulting in membrane depolarization (Sun et al., 2009). It is not clear to us which neurotransmitter is involved in the relay of the signal to the mitral cells. While CO_2 sensing via GC-D is an important sensory cue for many mammals, guanylyl cyclase-D became a nonfunctional pseudogene early in primate evolution and CO_2 is odorless to humans (Young et al., 2007).

Pheromone sensing through phospholipase C and transient potential channels

As we have mentioned, humans express five pheromone receptors (type V1R) which are detected in the olfactory epithelium (Duluc and Torello, 2003). Pheromones are "chemical substances released by one member of a species as a means of communication with another member, to their mutual benefit." Odorants can function as pheromones. As an example, the seasonally anestrous endocrine state of female goats is changed to the estrous state upon exposure to male scents. This so-called "male effect" is brought about by 4-ethyloctanal produced by the male head skin, received by olfactory receptors in the olfactory epithelium of female goats and leading to the release of gonadotropin-releasing hormone (GnRH) by the hypothalamus. This in turn is responsible for the release of luteinizing hormone in the anterior pituitary. Similar compounds, 6-ethyloctanal or 5-ethylheptane-2,4-dione, bind to receptors in the vomeronasal and bring about the same effect (Murata et al., 2014). Until today there are no examples of odorants that act as pheromones in humans (Meredith, 2001).

Microvillous vomeronasal neurons of rodents employ a phospholipase C (Plcb2) pathway and signal in a fashion that resembles light detection in the compound eye of invertebrates (discussed above). Receptor activation leads to the production of inositol-1,4,5-trisphosphate and diacylglycerol.

The G-protein involved is Gαi2 and Gαo, which do not interact with phospholipase C but instead the activating effect is brought about by Gβγ. This causes, by an as yet poorly defined mechanism, an increased conductance of the Trcp2 channel (Clapham, 2003). The increase in inward Na^+/Ca^{2+} current leads to membrane depolarization and this is translated into an increased neurotransmitter release. The axons of the vomeronasal sensory neurons are long and they connect with mitral cells in the glomeruli near the olfactory bulb (accessory olfactory system). As for the many pheromone receptors, the human homolog of the Trp2 channel is also a pseudogene.

PHEROMONE REVIEWS

- Zufall, F., Leinders-Zufall, T., 2007. Mammalian pheromone sensing. Curr. Opin. Neurobiol. 17, 483–489.
- Dulac, C., Torello, A.T., 2003. Molecular detection of pheromone signals in mammals: from genes to behavior. Nat. Rev. Neurosci. 4, 551–562.
- Meredith, M., 2001. Human vomeronasal organ function: a critical review of best and worst cases. Chem. Senses 26, 433–445.
- Buck, L.B., 2000. The molecular architecture of odor and pheromone sensing in mammals. Cell 100, 611–618.

The human vomeronasal organ has been the subject of some interest in the scientific literature and of considerable speculation in the popular science literature. Although further experimental work is required, current evidence does not support the notion that chemical communication occurs among humans. As such, we cannot speak of human pheromone signaling. Perhaps that color vision, which emerged at an evolutionary time where primates lost functionality of many of the pheromone receptor genes, has replaced pheromone control (apes and humans act by the looks and less by the smell).

THE GPCR SUPERFAMILY

The (metabotropic) receptors dealt with in this chapter and previous chapters are all members of the same GPCR superfamily. The feature that relates these receptors is the topological organization of the peptide chain, which in all traverses the membrane seven times (7 TM with helices 1–7), starting in the extracellular space and ending in the cytosol. The intracellular and extracellular loops (ECL) connecting the transmembrane helices vary in length between the different receptors. A topological diagram is depicted in Figure 5-20. The other essential feature that relates these receptors is their interaction with heterotrimeric GTP-binding proteins. In comparison with other major protein families, the level of sequence homology of this huge family is low. However, sequence alignments do reveal some shared traits, in particular, highly conserved amino acids in the transmembrane regions. These residues serve as an anchor point for a generic numbering of amino acids. Thus, in order to compare different receptors, compare their transmembrane helix-packing arrangement and their interaction with ligands, Ballesteros and Weinstein have proposed a method where the highest conserved residues are being denoted by their helix number and by number 50. For the surrounding residues, one counts downward or upward. For instance, arginine-51 (R51) in the first helix of the human β2-adrenoceptor is R1.50, aspartate-79 (D79) in the second is D2.50, arginine-131 in the DRY motif of the third helix is R3.50, and so on (see Figure 5-20). A highly conserved cysteine-191 in the second ECL, which forms a disulfide bridge with cysteine-106 in helix 3, is indicated by xlC2.50 (Ballesteros and Weinstein, 1995). We have employed generic numbering in Chapter 3 "Regulation of Muscle Contraction by Adrenoceptors," in the Figures dealing with the β-adrenoreceptor and binding of adrenaline or pharmacologically active ligands (Figures 3-4 and 3-10). We return to generic numbering for the global characterization of ligand-binding sites of different rhodopsin-type receptors later in this section.

Of the structures analyzed, a characteristic packing arrangement of the transmembrane helix bundle becomes apparent with a strong contact network as illustrated in Figure 5-20(c). With respect to the rhodopsin-type GPCRs, ligands make contact with helices-3, -5, -6, and -7. At the cytosolic side, G-protein α-subunits make contacts with H3, H5, H6, and ICL2 (Venkatakrishnan et al., 2013). Attachment of Gα requires a ligand-mediated outward movement of H6.

Five categories of GPCRs

The sequence homology within the GPCR superfamily is exceedingly limited. Indeed that they are all derived from a single common ancestor or whether they arose as the result of convergent evolutionary processes has been repeatedly discussed. Moreover, if divergence in the face of conserved architecture is evident for the membrane-spanning segment (helix bundle), it is even more obvious for the various extracellular ligand-binding domains. For example, within just one subfamily, the adhesion receptors (orphan receptors), the exposed segments vary in length from 7 up to 2800 residues (Ji et al., 1998).

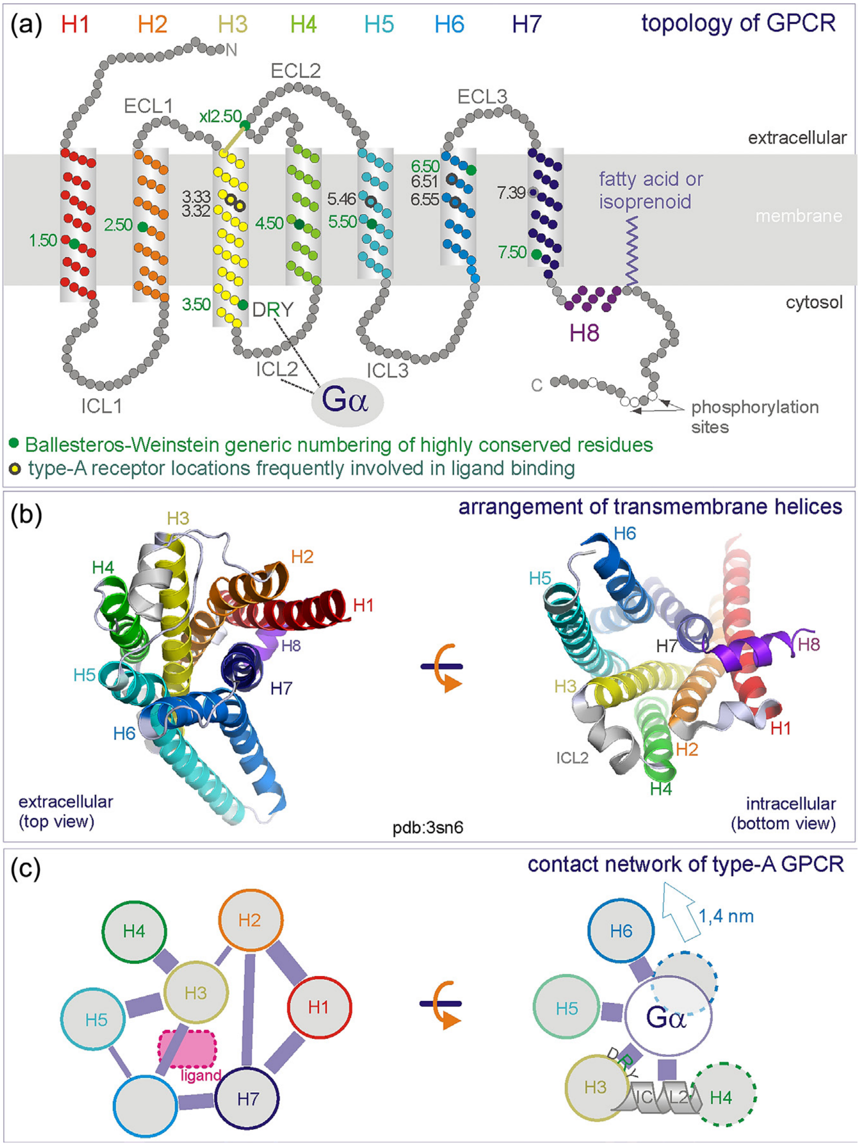


FIGURE 5-20 Membrane topology of G-protein-coupled receptors (GPCRs). (a) Schematic two-dimensional topology of a GPCR. Highly conserved residues, shown in green, serve as anchors for a generic numbering of the amino acid positions according to the method of Ballesteros–Weinstein. These residues are given two numbers, one the transmembrane helix to which they belong and second the number 50 (1.50, 2.50, etc.). As an example of the use of this system, we indicate the position of residues frequently involved in ligand binding (3.32, 3.33, 5.46, 6.51, 6.55, or 7.39). The nature of these residues varies of course between different receptors. (b). Top and bottom view of the three-dimensional packing arrangement of the seven transmembrane helices of the bovine β2-adrenoceptor. A short helix, extending from H7 at the cytosolic side, is denoted by H8. (c) Schematic representation of the contact network of the transmembrane helical segment of rhodopsin-type receptors (“top view” on the left, “bottom view” on the right). The lines between a pair of helices indicate the presence of consensus contacts and the thickness of the line is proportional to the number of amino acids involved. Note the strategic position of H3, connecting with four helices and making contact with both ligand and the G-protein α-subunit (DRY motif). The arrow (1.4 nm) indicates the movement of H6 necessary for binding of Galpha.

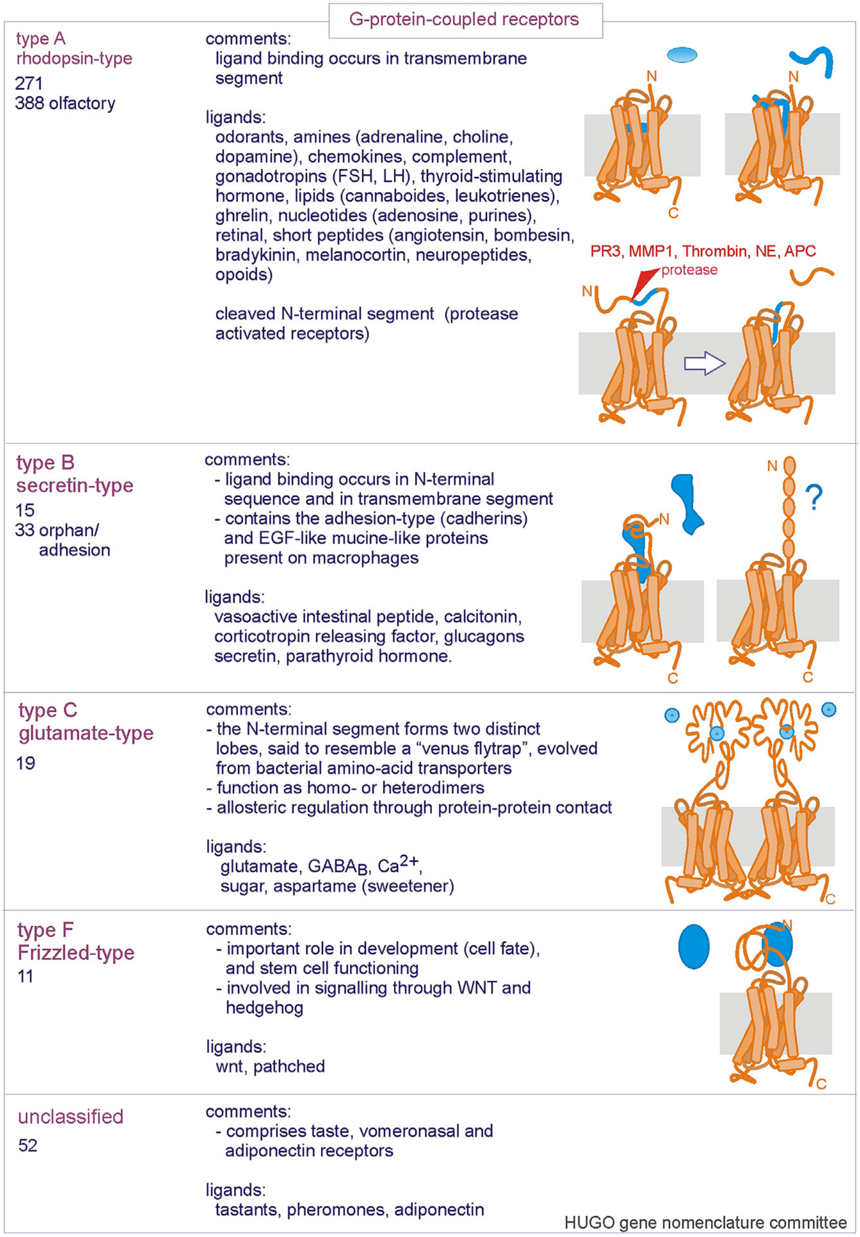
Currently, the HUGO gene nomenclature committee recognizes some 800 different genes (3.2% of the protein-coding genome) separated over four different categories plus a set of unclassified GPCRs. The classification is based on sequence comparison and the nature of the ligand. As already mentioned, these categories vary greatly in their extracellular N-termini. Category-A represents the rhodopsin-type (the largest by far because of the presence of 388 olfactory receptors), category-B the secretin-type, category-C the metabotropic glutamate-type, and category F represents the frizzled-type. Among the unclassified category, we find taste receptors, vomeronasal receptor type-1, adiponectin and orphan receptors (Figure 5-21). The missing letters, D and E, are reserved for two other categories: the fungal mating pheromone receptors and the *Dictyostelium discoideum* cAMP receptors, respectively (Fredriksson et al., 2003). It seems certain that the members within each category do share a common evolutionary origin.

It should be noted that many of the putative GPCRs are pseudogenes, counting some 563 for the odorants and pheromone receptors in the human genome. Moreover, numerous human receptors are still orphan, estimated at 150, lacking a known ligand and hence no known function. With respect to structure analysis, most studies concern category A, the rhodopsin-type.

An alternative classification system is proposed, similar to the above described but with an additional adhesion category, comprising 33 members (Bjarnadottir et al., 2004; Bjarnadóttir et al., 2009). The majority of this category qualifies as orphan receptor and in the HUGO gene nomenclature, its members are ranked among category-B orphans (proteins such as CELSR2, ERM1, BAI1, ETLD1, GPR65, GPR116, LPHN2, etc.). We briefly return to the seven membrane-spanning members of the cadherin adhesion molecules (CELSR1-3) in Chapter 11, "Signal Transduction to and from Adhesion Molecules."

Global description of the different GPCR categories

For the category-A receptors, the ligands are mainly of low molecular mass such as amines, nucleosides, small lipids, and short peptides (roughly 10 amino acids long) and they are located inside the transmembrane helix bundle. Dopamine penetrates roughly 1.4 nm into the transmembrane helix bundle, whereas IT1T (bound to CXCR4) remains close to the edge (Figure 5-22). Attachment involves both electrostatic and hydrophobic interactions (for detail about the binding pocket of the adrenoceptors, we refer to Chapter 3, Figures 3-4 and 3-10). Helices H3, H5, H6, and H7 make the major contributions and only a limited number of positions on these



G-protein-coupled receptors

type A rhodopsin-type 271 388 olfactory	comments: ligand binding occurs in transmembrane segment ligands: odorants, amines (adrenaline, choline, dopamine), chemokines, complement, gonadotropins (FSH, LH), thyroid-stimulating hormone, lipids (cannaboides, leukotrienes), ghrelin, nucleotides (adenosine, purines), retinal, short peptides (angiotensin, bombesin, bradykinin, melanocortin, neuropeptides, opoids) cleaved N-terminal segment (protease activated receptors)
type B secretin-type 15 33 orphan/ adhesion	comments: - ligand binding occurs in N-terminal sequence and in transmembrane segment - contains the adhesion-type (cadherins) and EGF-like mucine-like proteins present on macrophages ligands: vasoactive intestinal peptide, calcitonin, corticotropin releasing factor, glucagons secretin, parathyroid hormone.
type C glutamate-type 19	comments: - the N-terminal segment forms two distinct lobes, said to resemble a “venus flytrap”, evolved from bacterial amino-acid transporters - function as homo- or heterodimers - allosteric regulation through protein-protein contact ligands: glutamate, $GABA_B$, Ca^{2+}, sugar, aspartame (sweetener)
type F Frizzled-type 11	comments: - important role in development (cell fate), and stem cell functioning - involved in signalling through WNT and hedgehog ligands: wnt, pathched
unclassified 52	comments: - comprises taste, vomeronasal and adiponectin receptors ligands: tastants, pheromones, adiponectin

HUGO gene nomenclature committee

FIGURE 5-21 Different types of G-protein-coupled receptors, their peculiarities, and some of their ligands.

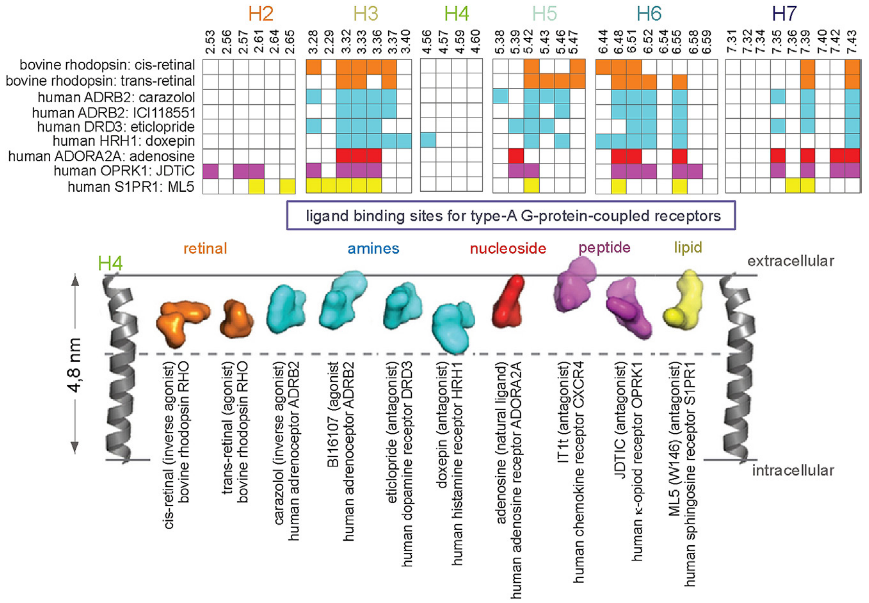

H2
2.53 2.56 2.57 2.61 2.64 2.65
H3
3.28 2.29 3.32 3.33 3.36 3.37 3.40
H4
4.56 4.57 4.59 4.60
H5
5.38 5.39 5.42 5.43 5.46 5.47
H6
6.44 6.48 6.51 6.52 6.54 6.55 6.58 6.59
H7
7.31 7.32 7.34 7.35 7.36 7.39 7.40 7.42 7.43
bovine rhodopsin: cis-retinal
bovine rhodopsin: trans-retinal
human ADRB2: carazolol
human ADRB2: ICI118551
human DRD3: eticlopride
human HRH1: doxepin
human ADORA2A: adenosine
human OPRK1: JDTiC
human S1PR1: ML5
ligand binding sites for type-A G-protein-coupled receptors
H4
retinal
amines
nucleoside
peptide
lipid
extracellular
intracellular
4,8 nm
cis-retinal (inverse agonist) bovine rhodopsin RHO
trans-retinal (agonist) bovine rhodopsin RHO
carazolol (inverse agonist) human adrenoceptor ADRB2
BI16107 (agonist human adrenoceptor ADRB2
eticlopride (antagonist) human dopamine receptor DRD3
doxepin (antagonist) human histamine receptor HRH1
adenosine (natural ligand) human adenosine receptor ADORA2A
IT1t (antagonist) human chemokine receptor CXCR4
JDTIC (antagonist) human κ-opiod receptor OPRK1
ML5 (W146) (antagonist) human sphingosine receptor S1PR1

FIGURE 5-22 Comparison of conserved amino acid positions and the depth of ligand-binding pockets for different G-protein-coupled receptors of the rhodopsin-type (category A). The top image compares the transmembrane helices residues that are involved in ligand attachment. Vertically, on the left, the identity of the receptor and horizontally the relative positions of the amino acids concerned (indicated by Ballesteros–Weinstein numbers). Note that totally different ligands interact with common positions in the transmembrane regions (for instance, all ligands interact with 3.32 and 3.33 and nearly all interact with 6.51 and 6.55). Naturally, the nature of the amino acids at these conserved positions varies (otherwise all binding pockets would fix the same ligand). The bottom image shows ligand penetration into the transmembrane helix bundle relative to the position of helix 4 (TM4). The histamine antagonist doxepin is buried deeply in HRH1, whereas the antagonist IT1T barely enters the transmembrane bundle of chemokine receptor CXCR4. The label on the top indicates the type of ligand (retinal, amines, etc.). *Image adapted with permission from* Venkatakrishnan et al. (2013).

helices are concerned. In Ballesteros–Weinstein numbering, positions 3.32, 3.33, 6.51, 7.39, and 7.43 play a key role in ligand attachment of most of the category-A receptors analyzed until today (Venkatakrishnan et al., 2013).

Protease-activated receptors also belong to this category but they stand apart in that the ligand is obtained through cleavage of their own N-terminal (Vu et al., 1991). Several proteases are concerned but thrombin takes central stage. Thrombin activates blood platelets causing them to aggregate within seconds. It also has numerous longer-term functions related to inflammation and tissue repair which are mediated by a wide range of cell types. Thrombin is a serine proteinase, an enzyme related to trypsin and chymotrypsin and also to acetylcholine esterase. It has a unique specificity, cleaving peptide chains between arginine and serine, only as they are embedded in particular peptide sequences. As with the other serine esterases, the proteolytic activity of thrombin can be inhibited by the organophosphorus compounds mentioned in Chapter 4, “Cholinergic Signaling and Muscle Contraction” and its action in stimulating blood platelets is also inhibited by compounds of this class. Thrombin binds to N-terminal segment of proteinase activated receptors PAR-1, -3, or 4 (the so-called “thrombin receptors”) (Dery et al., 1998; Kahn et al., 1998; Coughlin, 2000). By cleaving the N-terminal exodomain, a new N-terminus is revealed which itself acts as a tethered ligand interacting with the transmembrane helix bundle of the receptor. In addition to the tethered ligand, the cleaved 41 residue peptide acts as a strong agonist for other receptors on platelets (Carbourough et al., 1994; Zhang et al., 2012). Inappropriate thrombin activation, and subsequent blood clotting, lies at the heart of most heart attacks and strokes.

Although conventional in the sense that these PAR receptors are coupled to G-proteins (Gi, G12/13, and Gq are involved), there is the particular problem that their activation by proteolytic cleavage is necessarily irreversible. Of course, for the functioning of platelets there is no problem since stimulation initiates a sequence of events which terminates in their demise. For PAR receptors in other tissues, specialized mechanisms ensure their desensitization and removal (Trejo et al., 1998). In addition to the usual processes of receptor phosphorylation and endocytosis, these include cleavage of the tethered ligand in lysosomes. Because the receptors undergo cleavage both as a consequence of stimulation and again in the process of desensitization, resensitization of the system necessarily occurs by de novo protein synthesis.

In the epithelia of the upper intestine, PAR2 receptors confer protection against self-digestion by proteolytic enzymes through the production of prostaglandins. Similarly, in the bronchial airways, the presence of proteolytic enzymes released by inflammatory cells (mast cells) appears to be signaled by PAR receptors (Cocks et al., 1999).

For the category-B receptors, the binding sites for peptide hormones, such as secretin (27 amino acids), adrenocorticotropic hormone (ACTH, 38 amino acids), glucagon (29 amino acids), or corticotropin-releasing hormone (also known as corticoliberin and 41 amino acids long) comprise the N-terminal segment, exposed loops linking the transmembrane helices and a pocket inside the transmembrane helix bundle (Ploszak et al., 2008; Sakmar, 2011). With respect to the transmembrane segment, the packing arrangement of helices H1–5 of the corticotropin-releasing factor receptor 1 (CRHR1) is near identical to the category-A receptors (example of the D3 dopamine receptor) but differs for helices-6 and -7, which are bend outward. This creates a much wider cavity inside the transmembrane segment (Figure 5-23). Quite surprisingly, an antagonist of the corticotropin receptor (CP-376395) penetrates deeply into the protein, and binds a site normally inaccessible for natural ligands (Hollenstein et al., 2013). It remains to be studied whether these characteristics apply for other receptors of this category.

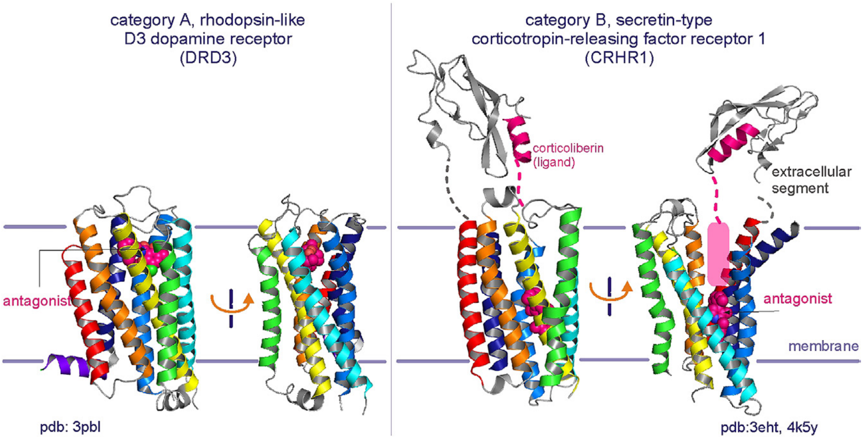


FIGURE 5-23 Comparison of the transmembrane helix-packing arrangements of a category-A (DRD3) and a category-B (CRHR1) receptor. The CRHR1 receptor is composed of two parts, a ligand-binding N-terminal extracellular segment (in gray) which binds the N-terminal section of corticoliberin (colored in hotpink) and a transmembrane segment. Note that the packing arrangement of helices 1, 2, 3, 4, and 5 is nearly identical between the two receptors, but helices-6 and -7 of CRHR1 make a steep outward bend at the level of the outer leaflet of the plasma membrane. This creates a much wider cavity in which we have projected the C-terminal section of corticoliberin. The antagonist of the DRD3 receptor binds at the expected higher upper part of the receptor, whereas the antagonist of CRH1 is deeply buried into the transmembrane helix bundle.

The category-B also harbors a subfamily with very large N-terminal extensions that contain motifs characteristic of cell surface adhesion molecules (Stacey et al., 2000). For example, the N-terminal extensions of the ERM1–3 group expressed predominantly in leukocytes, all contain multiple EGF-like domains. In addition, there is an RGD (arginine-glycine-aspartate) motif, a mucin-like stalk and a cysteine box. These appear to mediate cellular adhesion and for two members of this family, EMR2 and CD97, the cellular ligand has been identified as a sulfated glycosaminoglycan (Stacey et al., 2003). Interestingly, it is uncertain whether signaling of these receptors is conveyed through GTP-binding proteins nor even if it is mediated through the helix bundle domain. Included in this subcategory there are also the adhesion molecules that qualify as cadherins which play important roles in cellular junctions such as the zonula adherens and desmosome. We return to cadherin in Chapter 11 “Signal Transduction to and from Adhesion Molecules” (see Figure 11-10)

For the category-C, the sites of attachment for neurotransmitters (glutamate, $GABA_B$) and for Ca^{2+} are on specialized extracellular N-terminal extensions of up to 600 residues (Brown et al., 1993). Binding of Ca^{2+} causes a pincer-like conformational change in the lobes of the extended extracellular domain (resembling the movement of the Venus flytrap). This exposes residues which then interact with the transmembrane core of the receptor. In this way, the extracellular domain acts as an autoligand. The Ca^{2+}-sensing receptor confronts a particular problem since it has to sense and then respond to very small changes in Ca^{2+} concentration against a high basal level (2.5 mmol/l). The cells of the parathyroid gland react by secretion of parathyroid hormone whenever the concentration of circulating Ca^{2+} dips below about 2.2 mmol/l. Obviously, the affinity of the sensor has to be very low or it would be fully saturated at all times and under all conditions. On the other hand, the system must be sensitive to proportional changes in concentration that are minute compared with those sensed by conventional hormone receptors which react to changes ranging over orders of magnitude. The Ca^{2+} sensor is endowed with an extracellular domain which acts as a low-affinity chelator, binding or releasing Ca^{2+} as its concentration varies within the (extracellular) physiological range (Brown et al., 1993). It may operate as a dimer, the two components being joined by a disulfide bond between cysteine residues present in the extracellular domain. (A monomer–dimer equilibrium could underlie the special binding properties of this receptor (Pin et al., 2003).)

Category-F comprises exclusively the receptors for the Wnt family of ligands which play a role in the regulation of cell fate and cell polarity through a number of different routes, only one of which involves heterotrimeric G-proteins (Gαo) (Katanaev et al., 2005). We return to Wnt extensively in Chapter X "Adhesion Molecules in the Regulation of Cell Differentiation; Mainly About Wnt."

MULTIMEDIA RESOURCE

HUGO gene nomenclature committee: GPCR superfamily members
http://www.genenames.org/genefamilies/GPCR

Receptor activation mechanisms

Most of the members of the GPCRs signal through heterotrimeric G-proteins. Ligands must bring about conformational changes that facilitate the docking of a Gα-subunit at the cytosolic side of the transmembrane helix bundle. For rhodopsin-type receptors, this requires an outward movement of the H6 (Figure 5-20). Helices H3, harboring the conserved DRY motif, H5 and H6, as well as ICL2, play an important role in the attachment of the G-protein (for molecular detail return to Chapter 3, Figures 3-4–3-7). The receptor-bound Gα-subunit loses its

GDP which is replaced by GTP (GTPase exchange function of the active receptor). This in turn causes a functional dissociation of the heterotrimeric complex, allowing both Gα and Gβγ to seek effector proteins.

Studies with agonists, biased agonists, and reverse agonists have revealed that GPCR receptors occur in different conformational states, some of which prevent activation of G-proteins, others favor, and yet others may lead to binding of other proteins (Kahsai et al., 2011; Nygaard et al., 2013; Liu et al., 2012). Cases in point are the G-protein-regulated kinases which may directly bind to photoexcited rhodopsin (GRK1) and to the agonist-bound β-adrenoceptor (GRK6) so to cause phosphorylation of the cytoplasmic tail (Huang and Tesmer, 2011). Phosphorylation is followed by binding of members of the arrestin family, which for rhodopsin signals the end of signaling but for adrenoceptors signifies the start of a new cascade of events. Adrenoceptor-coupled arrestins (ARRB1, -B2) recruit protein kinases normally involved in growth factor signaling and bring about signaling pathways not normally recruited by G-proteins (Wisler et al., 2007). It is not sure whether or not similar activation mechanisms apply for the other categories of the GPRC superfamily. Although the secretin-type receptor CRHR1 manifests a similar transmembrane helix-packing arrangement at the cytosolic side of the transmembrane bundle (Figure 5-23), the amino acid composition of this part of the helices is quite different. For instance, the DRY motif is replaced by G(C/L)Y and we take this to mean that recruitment of Gα occurs by a different molecular mechanism (Hollenstein et al., 2013).

References

Angleson, J.K., Wensel, T.G., 1993. A GTPase-accelerating factor for transducin, distinct from its effector cGMP phosphodiesterase, in rod outer segment membranes. Neuron 11, 939–949.

Arendt, D., 2003. Evolution of eyes and photoreceptor cell types. Int. J. Dev. Biol. 47, 563–571.

Arshavsky, V.Y., Wensel, T.G., 2013. Timing is everything: GTPase regulation in phototransduction. Ophthalmol. Vis. Sci. 54, 7725–7733.

Arshavsky, V.Y., Lamb, T.D., Pugh, Jr. E.N., 2002. G-proteins and phototransduction. Annu. Rev. Physiol. 64, 153–187.

Baehr, W., Palszewski, K., 2009. Focus on molecules: guanylate cyclase-activating proteins (GCAPs). Exp. Eye Res. 89, 2–3.

Ballesteros, J.A., Weinstein, H., 1995. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosci. 25, 366–428.

Baud, O., Etter, S., Spreafico, M., Bordoli, L., Schwede, T., Vogel, H., Pick, H., 2011. The mouse eugenol odorant receptor: structural and functional plasticity of a broadly tuned odorant binding pocket. Biochemistry 50, 843–853.

Baylor, D.A., Lamb, T.D., Yau, K.W., 1979. Responses of retinal rods to single photons. J. Physiol. 288, 613–634.

Baylor, D.A., Nunn, B.J., Schnapf, J.L., 1984. The photocurrent, noise and spectral sensitivity of rods of the monkey *Macaca fascicularis*. J. Physiol. 357, 575–607.

Benton, R., 2009. Evolution and revolution in odor detection. Science 326, 382–383.

Bjarnadottir, T.K., Fredriksson, R., Höglund, P.J., et al., 2004. The human and mouse repertoire of the adhesion family of G-protein-coupled receptors. Genomics 84, 23–33.

Bjarnadóttir, T.K., Gloriam, D.E., Hellstrand, S.H., Kristiansson, H., Fredriksson, R., Schiöth, H.B., 2009. Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse. Genomics 88, 263–273.

Boron, W.F., Boulpaep, E.L., 2011. Medical Physiology, second ed. Elsevier.

Breer, H., Boekhoff, I., Tareilus, E., 1990. Rapid kinetics of second messenger formation in olfactory transduction. Nature 345, 65–68.

Bridges, C.D., Alvarez, R.A., 1987. The visual cycle operates via an isomerase acting on all-trans retinal in the pigment epithelium. Science 236, 1678–1680.

Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M.A., Lytton, J., Hebert, S.C., 1993. Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. Nature 366, 575–580.

Buck, L., Axel, R., 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175–187.

Carbourough, R.M., Naughton, M.A., Teng, W., Hung, D.T., Rose, J., Vu, T.K.H., Wheaton, V.I., Turck, C.W., Coughlin, S.R., 1994. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. J. Biol. Chem. 269, 32522–32527.

Chandrashekar, J., Hoon, M.A., Nicholas, J.P., Ryba, N.J.P., Zuker, C.S., 2006. The receptors and cells for mammalian taste. Nature 44, 288–294.

Cheever, M.L., Snyder, J.T., Gershburg, S., Siderovski, D.P., Harden, T.K., Sondek, J., 2008. Crystal structure of the multifunctional Gbeta5-RGS9 complex. Nat. Struct. Mol. Biol. 15, 155–162.

Chen, C.K., Burns, M.E., He, W., Wensel, T.G., Baylor, D.A., Simon, M.I., 2000. Slowed recovery of rod photoresponse in mice lacking the GTPases accelerating protein RGS9-1. Nature 64, 557–560.

Choe, H.W., Kim, Y.J., Park, J.H., Morizumi, T., Pai, E.F., Krauss, N., Hofmann, K.P., Scheerer, P., Ernst, O.P., 2011. Crystal structure of metarhodopsin II. Nature 471, 651–655.

Clapham, D.E., 2003. TRP channels as cellular sensors. Nature 426, 517–524.

Cocks, T.M., Fong, B., Chow, J.M., Anderon, G.P., Frauman, A.G., Goldie, R.G., Henry, P.K., Carr, M.J., Hamilton, J.R., Moffett, S., 1999. A protective role for protease-activated receptors in the airways. Nature 398, 156–160.

Cook, B., Bar-Yaacov, M., Cohen Ben-Ami, H., Goldstein, R.E., Paroush, Z., Selinger, Z., Minke, B., 2000. Phospholipase C and termination of G-protein-mediated signalling in vivo. Nat. Cell Biol. 2, 296–301.

Coughlin, S.R., 2000. Thrombin signalling and protease-activated receptors. Nature 407, 258–264.

Cygnar, K.D., Zhao, H., 2009. Phosphodiesterase 1C is dispensable for rapid response termination of olfactory sensory neurons. Nat. Neurosci. 12, 454–462.

Daemen, F.J., 1973. Vertebrate rod outer segment membranes. Biochim. Biophys. Acta 300, 255–288.

Dawson, T.M., Arriza, J.L., Jaworsky, D.E., et al., 1993. β-adrenergic receptor kinase-2 and β-arrestin-2 as mediators of odorant-induced desensitization. Science 259, 825–829.

Deretic, D., Wang, J., 2012. Molecular assemblies that control rhodopsin transport to the cilia. Vision Res. 75, 5–10.

Dery, O., Corvera, C.U., Steinhoff, M., Bunnett, N.W., 1998. Proteinase-activated receptors: novel mechanisms of signalling by serine proteases. Am. J. Phsyiol. 274C, 1429–1452.

Dolph, P.J., Ranganathan, R., Colley, N.J., Hardy, R.W., Socolich, M., Zuker, C.S., 1993. Arrestin function in inactivation of G protein-coupled receptor rhodopsin in vivo. Science 260, 1910–1916.

Dulac, C., Torello, A.T., 2003. Molecular detection of pheromone signals in mammals: from genes to behaviour. Nat. Rev. Neurosci. 4, 551–562.

Fain, G.L., Hardie, R., Laughlin, S.B., 2010. Phototransduction and the evolution of photoreceptors. Curr. Biol. 20, R114–R124.

Feiler, R., Bjornson, R., Kirschfeld, K., 1992. Ectopic expression of ultraviolet-rhodopsin in the blue photoreceptor cells of *Drosophila*: visual physiology and photochemistry of transgenic animals. J. Neurosci. 12, 3862–3868.

Feinstein, P., Mombaerts, P.A., 2004. A contextual model for axonal sorting into glomeruli in the mouse olfactory system. Cell 117, 817–831.

Firestein, S., Picco, C., Menini, A., 1993. The relation between stimulus and response in olfactory receptor cells of the tiger salamander. J. Physiol. 468, 1–10.

Firestein, S., 2004. A code in the nose. Sci. STKE 227, pe15.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.-G., Schioth, H.B., 2003. The G-protein-coupled receptors in the human genome form five main families: phylogenetic analysis, paralogon groups and fingerprints. Mol. Pharmacol. 63, 1256–1272.

Fülle, H.-J., Vassar, R., Foster, D.C., et al., 1995. A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. Proc. Natl. Acad. Sci. U.S.A. 92, 3571–3575.

Granovsky, A.E., Natochin, M., Artemyev, N.O., 1997. The γ subunit of rod cGMP-phosphodiesterase blocks the enzyme catalytic site. J. Biol. Chem. 272, 11686–11689.

Hardie, R.C., Postma, M., 2008. Phototransduction in microvillar photoreceptors of *Drosophila* and other invertebrates. In: Handbook of the Senses. Chapter 1.05, vol. 1. Elsevier.

Hardie, R.C., 2012. Phototransduction mechanisms in *Drosophila* microvillar photoreceptors. WIREs Membr. Transp. Signal 1, 162–187.

He, W., Cowan, C.W., Wensel, T.G., 1998. RGS9, a GTPase accelerator for phototransduction. Neuron 20, 95–102.

Hecht, S., Shlaer, S., Pirenne, M.P., 1942. Energy, quanta and vision. J. Gen. Physiol. 25, 819–840.

Heidelberger, R., Heinemann, C., Neher, E., Matthews, G., 1994. Calcium dependence of the rate of exocytosis in a synaptic terminal. Nature 371, 513–515.

Henderson, S.R., Reuss, H., Hardie, R.C., 2000. Single photon responses in *Drosophila* photoreceptors and their regulation by Ca^{2+}. J. Physiol. 524, 179–194.

Hollenstein, K., Kean, J., Bortolato, A., Cheng, R.K.Y., Dore, A.S., Jazayeri, A., Cooke, R.M., Weir, M., Marshall, F.H., 2013. Structure of class B GPCR corticotropin-releasing factor receptor 1. Nature 499, 438–483.

Hotta, Y., Benzer, S., 1969. Abnormal electroretinograms in visual mutants of *Drosophila*. Nature 222, 354–356.

Huang, C.C., Tesmer, J.J., 2011. Recognition in the face of diversity: interactions of heterotrimeric G- proteins and G protein-coupled receptor (GPCR)-kinases with activated GPCRs. J. Biol. Chem. 286, 7715–7721.

Huber, A., Sander, P., Paulsen, R., 1996. Phosphorylation of the InaD gene product, a photoreceptor membrane protein required for recovery of visual excitation. J. Biol. Chem. 271, 11710–11717.

Imamoto, Y., Shichida, Y., 2014. Cone visual pigments. Biochim. Biophys. Acta 1837 (5), 664–673. 2013-epubS0005-2728(13) 00146–1.

Ji, T.H., Grossmann, M., Ji, I., 1998. G protein-coupled receptors. I. Diversity of receptor ligand interactions. J. Biol. Chem. 273, 17299–17302.

Jones, D.T., Reed, R.R., 1989. Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. Science 244, 790–795.

Juilfs, D.M., Fülle, H.J., Zhao, A.Z., et al., 1997. A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. Proc. Natl. Acad. Sci. U.S.A. 94, 3388–3395.

Kahn, M.L., Zheng, Y.W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, J., Tam, C., Coughlin, S.R., 1998. A dual thrombin receptor system for platelet activation. Nature 394, 690–694.

Kahsai, A.W., Xiao, K., Rajagopal, S., Ahn, S., Shukla, A.K., Sun, J., Oas, T.G., Lefkowitz, R.J., 2011. Multiple ligand-specific conformations of the β2-adrenergic receptor. Nat. Chem. Biol. 7, 692–700.

Katanaev, V.L., Ponzielli, R., Semeriva, M., Tomlinson, A., 2005. Trimeric G protein-dependent frizzled signalling in *Drosophila*. Cell 120, 111–122.

Komolow, K.E., Senin, I.I., Kovaleva, N.A., Christoph, M.P., Churumova, V.A., Grigoriev, I.I., Akhtar, M., Philippov, P.P., Koch, K.-W., 2009. Mechanism of rhodopsin kinase regulation by recoverin. J. Neurochem. 110, 72–79.

Kühne, W., 1878. On the Photochemistry of the Retina and on Visual Purple. Macmillan, London.

Lagnado, L., Baylor, D.A., 1992. Signal flow in visual transduction. Neuron 8, 995–1002.

Lamb, T.D., Pugh, Jr. E.N., 2004. Dark adaptation and the retinoid cycle of vision. Prog. Retin. Eye Res. 23, 307–380.

Leinders-Zufall, T., Rand, M.N., Shepherd, G.M., Greer, C.A., Zufall, F., 1997. Calcium entry through cyclic nucleotide-gated channels in individual cilia of olfactory receptor cells: spatiotemporal dynamics. J. Neurosci. 17, 4136–4148.

Liebman, P.A., Pugh, E.N., 1979. The control of phosphodiesterase in rod disk membranes; kinetics, possible mechanisms and significance for vision. Vision Res. 19, 375–380.

Lim, S., Peshenko, I.V., Dizhoor, A.M., Ames, J.B., 2013. Structural insights for activation of retinal guanylate cyclase by GCAP1. PLoS One 8, e81822.

Liu, W., Clark, W.A., Sharma, P., Northrup, J.K., 1998. Mechanism of allosteric regulation of the rod cGMP phosphodiesterase activity by the helical domain of transducin α subunit. J. Biol. Chem. 273, 34284–34292.

Liu, M., Parker, L.L., Wadzinski, B.E., Shieh, B.H., 2000. Reversible phosphorylation of the signal transduction complex in *Drosophila* photoreceptors. J. Biol. Chem. 275, 12194–12199.

Liu, J.J., Horst, R., Katritch, V., Stevens, R.C., Wuthrich, K., 2012. Biased signalling pathways in β2-adrenergic receptors characterized by 19F-NMR. Science 335, 1106–1110.

Mashukova, A., Spehr, M., Hatt, H., Neuhaus, EM., 2006. β-arrestin2-mediated internalization of mammalian odorant receptors. J. Neurosci. 26, 9902.

Matsunami, H., Amrein, H., 2003. Taste and pheromone perception in mammals and flies. Genome Biol. 4, 220.

Meredith, M., 2001. Human vomeronasal organ function: a critical review of best and worst cases. Chem. Senses 26, 433–445.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., Axel, R., 1996. Visualizing an olfactory sensory map. Cell 87, 675–686.

Morgans, C.W., 2000. Neurotransmitter release at ribbon synapses in the retina. Immunol. Cell Biol. 78, 442–446.

Murata, T., Tamogami, S., Itou, M., Ohkubo, Y., Wakabayshi, Y., Watanabe, H., Okamura, H., Takeuchi, Y., Mori, Y., 2014. Identification of an olfactory signal molecule that activates the central regulation of reproduction in goats. Curr. Biol. 24, 1–6.

Nakamura, T., Gold, G.H.A., 1987. Cyclic nucleotide-gated conductance in olfactory receptor cilia. Nature 325, 442–444.

Nei, M., Niimura, Y., Nozawa, M., 2008. The evolution of animal chemosensory receptor gene repertoires: role of chance and necessity. Nat. Rev. Genet. 9, 951–963.

Nishiguchi, K.M., Sandberg, M.A., Kooijman, A.C., Martemyanov, K.A., Pott, J.W.R., Hagstrom, S.A., Arshavsky, V.Y., Berson, E.L., Dryja, T.P., 2004. Defects in RGS9 or its anchor R9AP in patients with slow photoreceptor deactivation. Nature 427, 75–78.

Nygaard, R., Zou, Y., Dror, R.O., Mildorf, T.J., Arlow, D.H., et al., 2013. The dynamic process of β2-adrenergic receptor activation. Cell 152, 532–542.

O'Connor, S., Jacob, T.J.C., 2008. Neuropharmacology of the olfactory bulb. Curr. Mol. Pharmacol. 1, 181–190.

Pace, U., Hanski, E., Salomon, Y., Lancet, D., 1985. Odorant sensitive adenylate cyclase may mediate olfactory reception. Nature 316, 255–258.

Pak, W.L., Grossfield, J., White, N.V., 1969. Non-phototactic mutants in a study of vision in *Drosophila*. Nature 222, 351–354.

Pappone, M.C., Hurley, J.B., Bourne, H.R., Stryer, L., 1982. Functional homology between signal-coupling proteins. Cholera toxin inactivates the GTPase activity of transducin. J. Biol. Chem. 257, 10540–10543.

Pin, J.-P., Galvez, T., Prézau, L., 2003. Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. Pharmacol. Ther. 98, 325–354.

Ploszak, A.A., Parker, N.R., Suino-Powell, K., Xu, H.E., 2008. Molecular recognition of corticotrophin-releasing factor by its G-protein-coupled receptor CRFR1. J. Biol. Chem. 283, 32900–32912.

Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., et al., 2011. Crystal structure of the β2-adrenergic receptor-Gs protein complex. Nature 477, 549–555.

Restrepo, D., 2005. The ins and outs of intracellular chloride in olfactory receptor neurons. Neuron 45, 481–482.

Rodieck, R.W., 1998. The First Steps in Seeing. Massachussetts: Sinauer Associates, Sunderland.

Room, A., 1999. Brewer's Dictionary of Phrase and Fable. Cassell, London.

Sagoo, M.S., Lagnado, L., 1997. G-protein deactivation is rate-limiting for shut-off of the phototransduction cascade. Nature 389, 392–395.

Sakmar, T.P., 2011. Clicking class B GPCR ligands. Nat. Chem. Biol. 7, 500–501.

Shichida, Y., Masuyama, T., 2009. Evolution of opsins and phototransduction. Phil. Trans. R. Soc. B 364, 2881–2895.

Silbering, A.F., Benton, R., 2010. Ionotropic and metabotropic mechanism in chemoreception: "chance or design". EMBO Rep. 11, 173–179.

Sinnarajah, S., Dessauer, C.W., Srikumar, D., Chen, J., Yuen, J., Yilma, S., Dennis, J.C., Morrison, E.E., Vodyanoy, V., Kehrl, J.H., 2001. RGS2 regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III. Nature. 409, 1051–1055. http://www.nature.com.gate2.inist.fr/nature/journal/v409/n6823/full/4091051a0.html-a1.

Slep, K.C., Kercher, M.A., He, W., Cowan, C.W., Wensel, T.G., Sigler, P.B., 2001. Structural determinants for regulation of phosphodiesterase by a G protein at 2.0 A. Nature 409, 1071–1077.

Sokolov, M., Lyubarsky, A.L., Strissel, K.J., et al., 2002. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. Neuron 34, 96–106.

Sommer, M.E., Hofmann, K.P., Heck, M., 2012. Distinct loops in arrestin differentially regulate ligand binding within the GPCR opsin. Nat. Commun. 3, 995.

Song, Y., Cygnar, K.D., Sagdullaev, B., Valley, M., Hirsh, S., Stephan, A., Reisert, J., Zhao, H., 2008. Ca^{2+}/calmodulin-mediated fast desensitization by the B1b subunit of the CNG channel affects response termination but not sensitivity to recurring stimulation in olfactory sensory neurons. Neuron 58, 374–386.

Stacey, M., Lin, H.-H., Gordon, S., McKnight, A.J., 2000. LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. Trends Biochem. Sci. 25, 284–288.

Stacey, M., Chang, G.-W., Davies, J.Q., Kwakkenbos, M.J., Sanderson, R.D., Gordon, S., Lin, H.-H., 2003. The epidermal growth factor-like domains of the human ERM2 receptor mediate cell attachment through chondroitin sulphate glycosaminoglycans. Blood 102, 2916–2924.

Stephan, A.B., Shum, E.Y., Hirsh, S., Cygnar, K.D., Reisert, J., Zhao, H., 2009. ANO2 is the cilial calcium-activated chloride channel that may mediate olfactory amplification. Proc. Natl. Acad. Sci. U.S.A. 106, 11776–11781.

Stephan, A.B., Tobochnik, S., Dibattista, M., Reisert, J., Zhao, H., 2011. The Na^{+}/Ca^{2+} exchanger NCKX4 governs termination and adaptation of the mammalian olfactory response. Nat. Neurosci. 15, 131–137.

Stephen, R., Berat, G., Golczak, M., Palczewski, K., Carlos-Sousa, M., 2007. Stabilization function for myristoyl group revealed by the crystal structure of a neuronal calcium sensor, guanylate cyclase-activating protein 1. Structure 15, 1392–1402.

Struts, A.V., Salgado, G.F.J., Martinez-Mayorga, K., Brown, M.F., 2011. Retinal dynamics underlie its switch from inverse agonist to agonist during rhodopsin activation. Nat. Struct. Mol. Biol. 18, 392–394.

Sudhof, T.C., 2013. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. Neuron 80, 675–690.

Sun, L., Wanga, H., Hu, J., Han, J., Matsunami, H., Luo, M., 2009. Guanylyl cyclase-D in the olfactory CO_2 neurons is activated by bicarbonate. Proc. Natl. Acad. Sci. U.S.A. 106, 2041–2046.

Takeuchi, H., Kurahashi, T., 2005. Mechanism of signal amplification in the olfactory sensory cilia. J. Neurosci. 30, 11084–11091.

Trejo, J., Hammes, S.R., Coughlin, S.R., 1998. Termination of signalling by protease-activated receptor-1 is linked to lysosomal sorting. Proc. Natl. Acad. Sci. U.S.A. 95, 13698–13702.

Trudeau, M.C., Zagotta, W.N., 2002. Mechanism of calcium/calmodulin inhibition of rod cyclic nucleotide-gated channels. Proc. Natl. Acad. Sci. U.S.A. 99, 8424–8429.

Tsunoda, S., Sierralta, J., Sun, Y.M., Bodner, R., Suzuki, E., Becker, A., Socolich, M., Zuker, C.S., 1997. A multivalent PDZ domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388, 243–249.

Valeton, J.M., van Norren, D., 1983. Light adaptation of primate cones; an analysis based on extracellular data. Vision Res. 23, 1539–1547.

Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F., Babu, M.M., 2013. Molecular signatures of G-protein-coupled receptors. Nature 494, 185–194.

Vocke, K., Dauner, K., Hahn, A., Ulbrich, A., Broecker, J., Keller, S., Frings, S., Möhrlen, F., 2013. Calmodulin-dependent activation and inactivation of anoctamin calcium-gated chloride channels. J. Gen. Physiol. 142, 381–404.

Vu, T.K., Hung, T.D., Wheaton, V.I., Coughlin, S.R., 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64, 1057–1068.

Wei, J., Zhao, A.Z., Chan, G.C., et al., 1998. Phosphorylation and inhibition of olfactory adenylyl cyclase by CaM kinase II in Neurons: a mechanism for attenuation of olfactory signals. Neuron 21, 495–504.

Wheeler, G.L., Matuto, Y., Bitensky, M.W., 1977. Light-activated GTPase in vertebrate photoreceptors. Nature 269, 822–824.

Wisler, J.W., DeWire, S.M., Whalen, E.J., Violin, J.D., Drake, M., Ahn, S., Shenoy, S.K., Lefkowitz, R.J., 2007. A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. Proc. Natl. Acad. Sci. 104, 16657–16662.

Wong, S.T., Trinh, K., Hacker, B., et al., 2000. Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron 27, 487–497.

Yan, C., Zhao, A.Z., Bentley, J.K., Louchney, K., Ferguson, K., Beavo, J., 2003. Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. Proc. Natl. Acad. Sci. 92, 9677–9681.

Young, J.M., Waters, H., Dong, C., Fulle, H.J., Liman, E.R., 2007. Degeneration of the olfactory guanylyl cyclase D gene during primate evolution. PLoS One 2, e884.

Young, R.W., 1967. The renewal of photoreceptor cell outer segments. J. Cell Biol. 33, 61–72.

Zhang, H., Constantine, R., Vorobiev, S., Chen, Y., Seetharaman, J., Janet Huang, Y., Montelione, G.T., Gerstner, C.D., Davis, M.W., Inana, G., Whitby, G.F., Jorgensen, E.M., Hill, C.P., Tong, L., Baehr, W., 2011. UNC119 is required for G-protein trafficking in sensory neurons. Nat. Neurosci. 14, 874–880.

Zhang, C., Srinivasan, Y., Arlow, D.H., Fung, J., Palmer, D., Zheng, Y., Green, H.F., Pandey, A., Dror, R.O., Shaw, D.E., Weis, W.I., Coughlin, S.R., Kobilka, B.K., 2012. High resolution crystal structure of human proteinase-activated receptor 1. Nature 492, 387–392.

Zufall, F., Munger, S.D., 2001. From odor and pheromone transduction to the organization of the sense of smell. Trends Neurosci. 24, 191–193.

CHAPTER

6

Intracellular Calcium

Calcium is an abundant element. It constitutes 3% of the Earth's crust and it is prominent throughout the biosphere. It is present in fresh and sea water at levels that range from micromolar to millimolar. Ca^{2+} ions and Ca^{2+}-binding proteins are essential for many biochemical processes in prokaryotic and eukaryotic cells. In this chapter, we show that cells have obtained numerous Ca^{2+}-binding proteins that control a wide range of signaling mechanisms. We show how Ca^{2+} is studied and we place the subject of how cells deal with Ca^{2+} in the context of exocytosis and cell migration.

A NEW SECOND MESSENGER IS DISCOVERED

A number of the more enduring truths of science have been discovered when a failure of vigilance is coupled with uncommon perspicacity. Sydney Ringer's discovery of a requirement for calcium in a biological process must surely be placed in this category (Ringer, 1883, 1884).

Henry Dale's account

> Ringer was a physician to University College Hospital, and, in such time as he could spare from his practice, one of the pioneers of pharmacological research in this country. In his early experiments he had found that a solution containing only pure sodium chloride, common salt, in the proportion in which it is present in the serum of frog's blood would keep the beat of the heart in action for only a short time, after which it weakened and soon stopped. And then suddenly the picture changed: apparently the same pure salt solution would now maintain the heart in vigorous activity for many hours. Ringer was puzzled, and thought for a time that the difference must be due to a change in the season of the year—until he discovered what had really happened. Being busy with other duties, he had trusted the preparation of the solutions to his laboratory boy, one Fielder; and as Fielder himself, who I knew as an ageing man, explained to me, he didn't see the point of spending all that time distilling water for Dr Ringer, who wouldn't notice any difference if the salt solution was made up with

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> water straight out of the tap. But, as we have seen, Ringer did notice the difference; and when he discovered what had happened he did not merely become angry and insist on having distilled water for his saline solution, he took full advantage of the opportunity which accident had thus offered him and soon discovered that water from the tap, supplied then to North London by the New River Water Company, contained just the right small proportion of calcium ions to make a physiologically balanced solution with his pure sodium chloride….

Hints of a wider role for calcium soon followed with Locke's demonstration (Locke, 1894) that removal of calcium could block the transmission of impulses at the neuromuscular junction in a frog Sartorius-muscle preparation. The realization that this requirement for calcium is due to its role in controlling the secretion of a chemical messenger (neurotransmitter) had to wait nearly 50 years (Mann et al., 1939; Harvey and MacIntosh, 1940).

The direct introduction of Ca^{2+} ions into muscle fibers to cause contraction was first reported by Kamada and Kinoshita (1943). At this time Japan and the USA were at war, and as a result of the breakdown of communication, the credit for this finding has generally been ascribed to the Americans, Heilbrunn and Wiercinsky. They reported their results four years later (Heilbrunn and Wiercinsky, 1947). Other cations such as Na^+, K^+, and Mg^{2+} were found to be without effect. It had been Heilbrunn's contention, at that time at least, that the effect of calcium was on the "general colloid properties of the protoplasm" and that the effects of ions on isolated proteins would lack biological relevance (Heilbrunn, 1940). However, Otto Loewi who attended Heilbrunn's presentation at the New York Academy of Sciences was heard to growl "Kalzium ist alles" (return to Chapter 1, "Prologue"). So it remained, until the biochemical understanding of signaling mechanisms became more developed, taking in cyclic AMP, GTPases, tyrosine kinases, PI 3 kinases, and much more (see Chapter 2, "An Introduction to Signal Transduction"). Even with his foresight however, had Loewi lived to the end of the century, he would surely have been astounded by the prominence of calcium in contemporary biology.

In the previous chapters, we have seen how the idea of second messengers followed from the work of Sutherland and Rall (Sutherland, 1972) showing that the generation of cyclic AMP represents the essential link between membrane events and the metabolic process in the signaling of glycogenolysis. Reflecting this history, cyclic AMP was said to be the first second messenger and Ca^{2+} the second. In fact, elevation of cytosol Ca^{2+} is more ubiquitous than is the elevation of cyclic AMP, and it regulates a very diverse range of activities, including secretion, muscle contraction, fertilization, gene transcription, and cell proliferation (return to Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors" and Chapter 4 "Cholinergic Signaling and Muscle Contraction").

FREE, BOUND, AND TRAPPED Ca^{2+}

In the biological world, Ca^{2+} may be considered to exist in three main forms: trapped, bound, and free. In vertebrates, calcified tissues such as bones and teeth account for the major proportion of body calcium (mineralized tissues). Within these tissues calcium is trapped in mineral form (with phosphate) as hydroxyapatite ($Ca_5(PO_4)_3(OH)$). Apart from its structural function, bone provides a reservoir of slowly exchangeable calcium that can be mobilized, when needed, to maintain a steady extracellular concentration (Figure 6-1).

The total amount of calcium in extracellular fluid, in comparison with the mineralized tissues, is very small (mM range) and of this, only about half can be regarded as free. The remainder is bound mostly to proteins in the extracellular milieu. Within cells, the concentration of free Ca^{2+} inside the cytosolic compartment is still lower (nM range), by about four orders of magnitude (Figure 6-1). The cell organelles may contain higher levels of free Ca^{2+}, in particular the endoplasmic reticulum and endo/lysosomes (high μM range). In this chapter, we focus on cellular proteins that bind Ca^{2+} and which serve different roles. As we will learn some of the proteins simply sequester Ca^{2+}, but most of them are components of Ca^{2+}-regulated signaling pathways. For some proteins the structural arrangement for Ca^{2+} binding is flexible and this affects "on" and "off" kinetics of binding.

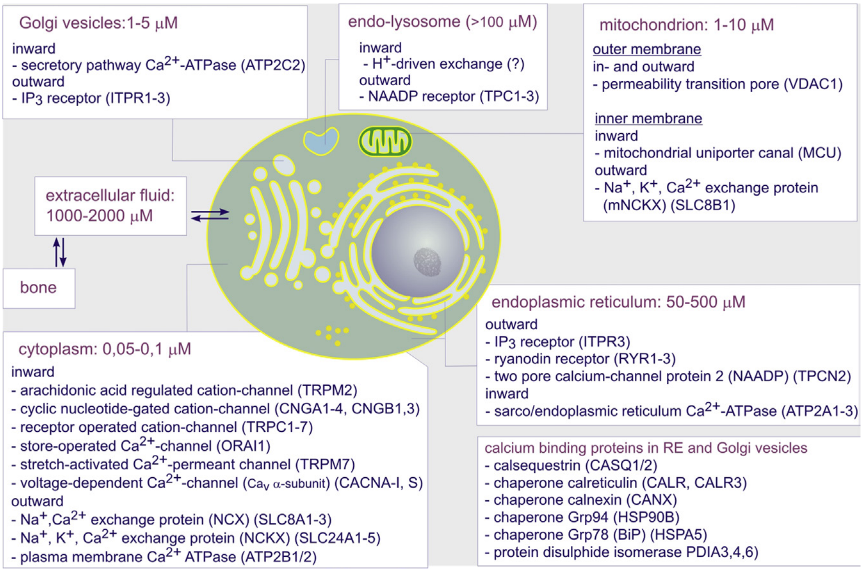

Golgi vesicles:1-5 μM
inward
- secretory pathway Ca2+-ATPase (ATP2C2)
outward
- IP3 receptor (ITPR1-3)
endo-lysosome (>100 μM)
inward
- H+-driven exchange (?)
outward
- NAADP receptor (TPC1-3)
mitochondrion: 1-10 μM
outer membrane
in- and outward
- permeability transition pore (VDAC1)
inner membrane
inward
- mitochondrial uniporter canal (MCU)
outward
- Na+, K+, Ca2+ exchange protein (mNCKX) (SLC8B1)
extracellular fluid: 1000-2000 μM
bone
endoplasmic reticulum: 50-500 μM
outward
- IP3 receptor (ITPR3)
- ryanodin receptor (RYR1-3)
- two pore calcium-channel protein 2 (NAADP) (TPCN2)
inward
- sarco/endoplasmic reticulum Ca2+-ATPase (ATP2A1-3)
cytoplasm: 0,05-0,1 μM
inward
- arachidonic acid regulated cation-channel (TRPM2)
- cyclic nucleotide-gated cation-channel (CNGA1-4, CNGB1,3)
- receptor operated cation-channel (TRPC1-7)
- store-operated Ca2+-channel (ORAI1)
- stretch-activated Ca2+-permeant channel (TRPM7)
- voltage-dependent Ca2+-channel (Cav α-subunit) (CACNA-I, S)
outward
- Na+,Ca2+ exchange protein (NCX) (SLC8A1-3)
- Na+, K+, Ca2+ exchange protein (NCKX) (SLC24A1-5)
- plasma membrane Ca2+ ATPase (ATP2B1/2)
calcium binding proteins in RE and Golgi vesicles
- calsequestrin (CASQ1/2)
- chaperone calreticulin (CALR, CALR3)
- chaperone calnexin (CANX)
- chaperone Grp94 (HSP90B)
- chaperone Grp78 (BiP) (HSPA5)
- protein disulphide isomerase PDIA3,4,6)

FIGURE 6-1 Approximate levels of free Ca^{2+} inside and outside the cell. Note the steep gradients between the extracellular environment, mM range, the different organelles, μM range, and the cytosol, which keeps Ca^{2+} at a very low nM range. Numerous pumps (active), exchangers (secondary active), and channels (passive transport) are involved in the distribution of Ca^{2+}.

Co-transporters, exchangers, and pumps use flexible binding sites to shuttle Ca^{2+} out of the cell or into a subcellular compartment (endoplasmic reticulum or Golgi vesicles).

The term ligand has two meanings. Chemists first coined the term to describe an electron-donating group that forms coordination complexes with metal ions. For instance, glutamates, asparagines, and aspartates are ligands in the Ca^{2+}-binding proteins. In pharmacology, the term ligand refers to a first messenger, and its various analogues (agonists, inverse agonists, and antagonists) that bind a receptor.

Cellular calcium-binding proteins possess different domains or contain short amino acid motifs that trap the ion with negatively charged oxygen groups, most often provided by the side chains of aspartate and glutamate. Water may also contribute to the coordination (Figure 6-2). Ca^{2+} has seven coordination sites whereas Mg^{2+}, another divalent metal abundantly present in the cytosol (mM range), has six. The coordination geometry of Ca^{2+} is asymmetric and that of Mg^{2+} is symmetric. Ca^{2+}-binding motifs make the distinction between the two metals and show much higher affinity for Ca^{2+} than for Mg^{2+} (with one or two exceptions, some EF-hands prefer Mg^{2+} rather than Ca^{2+} (Houdusse and Cohen, 1996). A long list of Ca^{2+}-binding proteins is shown in Figure 6-3.

Chelators of metals which are employed in industry and in research, such as BAPTA, EDTA, and EGTA, work by the same principle; they are small molecules that offer a set of coordination sites, hexa- or octahedral, which bind a wide range of metals.

CHELATING AGENTS

The word **chelate** is derived from *chela*, meaning a claw of a crab or lobster in Latin (Figure 6-2). Chelating agents are synthetic compounds that consist of a cage composed of several ligand atoms. Such multidentate ("multi-toothed") assemblies offer a set of coordination sites that fixes metals. EDTA (ethylenediaminetetra–acetic acid) forms strong compounds with Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Co^{3+}, and Ca^{2+} whereas the larger molecule EGTA (ethylene glycol tetraacetic acid) is one of the most specific Ca^{2+} chelators. This molecule possesses four carboxyl groups and two ether oxygen groups and exhibits a 10^5-fold selectivity for Ca^{2+} over Mg^{2+}. EGTA and similar compounds such as BAPTA are used in the laboratory as Ca^{2+} buffers to control the concentration of free Ca^{2+} in experiments.

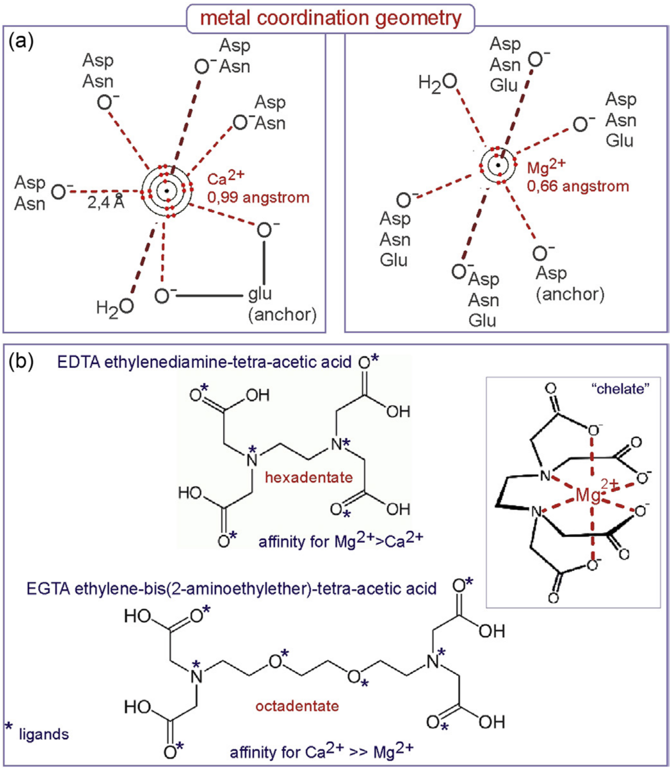


FIGURE 6-2 **Metal coordination geometry and metal chelators (EGTA and EDTA).** (a) Metal coordination sites of proteins employ predominantly aspartate, asparagine, and glutamate as coordinating ligands. Their negatively charged oxygen (side chain) electrostatically interacts with the positively charged metal. The size (number of electron orbits) and the coordination geometry for Ca^{2+} and Mg^{2+} are different and metal coordination sites in proteins distinguish between the two. (b) Metal chelators, such as EDTA and EGTA, work by the same principle; they offer numerous coordination sites, six and eight, respectively, that bind metal ions. EGTA binds Ca^{2+} with 10^5-fold higher affinity than Mg^{2+} and is considered Ca^{2+} selective.

CYTOSOLIC Ca^{2+} IS KEPT LOW

The cytosolic free Ca^{2+} concentration is kept low, in the order of 0.05–0.1 μM, and this is assured by active transport. Most of the Ca^{2+} is kept outside the cell but a small proportion is stored in intracellular compartments; the endoplasmic reticulum (named sarcoplasmic reticulum for muscle tissue), Golgi vesicles, endosomes, lysosomes, and mitochondria. There are three types of Ca^{2+}-ATPases that remove Ca^{2+} from the cytosol. They are localized in the plasma membrane (ATP2B), the endoplasmic reticulum (ATP2A), and the Golgi apparatus (ATP2C). Extrusion from the cytosol is also enabled by plasma membrane exchangers, either Ca^{2+}/Na^+ (SLC8A) or Ca^{2+}, K^+, Na^+ exchangers (SLC24A) and by and electrical

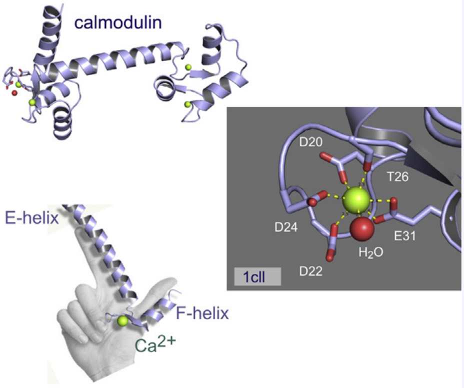
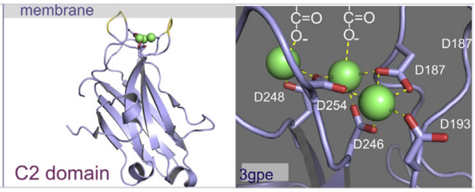
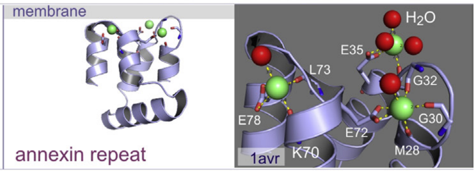
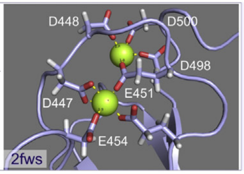
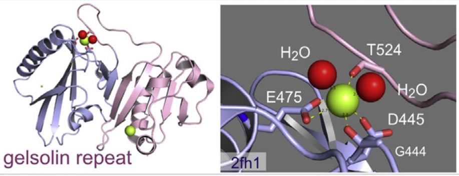
protein	Ca^{2+} binding domain or motif
α-actinin (ACTN 1-4) calcium integrin binding protein (CIB1) calcium binding protein (CABP 1-7) calcineurin B (PPP3R 1,2) calmodulin (CALM 1-3) calpain (CAPN 1-14) diacylglycerol kinase (DGKA A,B,G) filaggrin (FLG) guanyl cyclase activating (GUCA1 A-C) Kv channel-interacting protein (KCNIP1) mitochondrial transport subunit (MICU1/2) parvalbumin (PVALB) phospholipase C (PLC) ζ recoverin (RCVRN) S100 (A1-16, B,G,P,Z) spectrin (SPTAN1) stromal interaction molecule (STIM 1,2) troponin C (TNNC 1,2)	 calmodulin E-helix F-helix Ca^{2+} D20 T26 D24 E31 H_2O D22 1cll EF hand
cytosolic phospholipase A2 (PLA2G4A) phospholipase C (PLC) δ,ζ protein kinase C (PRKC) α,β1,β2,γ, δ, ε,τ) synaptotagmin (SYT 1-16)	 membrane C=O O- C=O O- D187 D187 D248 D254 D193 D246 3gpe C2 domain
annexin (ANXA1-11,13)	 membrane H_2O E35 L73 G32 E78 G30 E72 1avr K70 M28 annexin repeat
calnexin (CANX) calreticulin (CALR, CALR3) calsequestrin (CASQ 1,2) Grp78, Grp84 (HSP)	P-domain P- and C-domain charged protein surface - -
Ca^{2+}-pumps (ATP2A, 2B, 2C) Na^+/Ca^{2+} exchangers (SLC8 A1-3, B1) $Na^+/Ca^{2+}/K^+$ exchangers (SLC24 A1-5) large conductance K^+-channel (KCNMA1) voltage-gated Ca^{2+} channels (CACNA1)	- - Calx-β motif → - - Ca^{2+}-bowl - - 
adseverin (SCIN) gelsolin (GSN)	 gelsolin repeat

FIGURE 6-3 Examples of cellular Ca^{2+}-binding proteins and the structural organization of their Ca^{2+}-coordination sites. The proteins have been categorized by the Ca^{2+}-binding domains or motifs that they carry. We recognize proteins carrying a set of EF-hands (often four), a C2 domain, multiple annexin repeats, or gelsolin repeats. The Na^+/Ca^{2+} exchanger contains a CalX-β motif. A number of proteins that reside in the endoplasmic reticulum bind Ca^{2+} with as yet poorly defined motifs. Note that Ca^{2+} normally requires seven coordination sites, which are not always provided by the proteins. The remaining electrostatic interactions are supplemented by water or acidic phospholipids. Ca^{2+} is represented by lime-green spheres and water by red spheres. Pdb: 1cll, 3gpe, 1avr, 2fws, 2fh1.

gradient-driven Ca^{2+} channel in the inner membrane of the mitochondrion (MCU) (Kirichok et al., 2003). The rather high affinity of Ca^{2+} pumps, $K_D \sim 0.2\,\mu mol/l$ and Ca^{2+}/Na^+ exchangers, $K_D \sim 0.5\,\mu mol/l$ enables these transporters to bring resting levels down to a level of 0.1 μM (100 nM).

Entry of extracellular Ca^{2+} into the cytosol occurs passively, driven by both a chemical and an electrical gradient, through a large variety of nonspecific cation channels (Ca^{2+}-permeant channels). Their conductivity is controlled by a variety of compounds such as arachidonic acid, ADP-ribose, nicotinamide adenine dinucleotide (NAD), cyclic nucleotides, membrane receptors, the Ca^{2+} content of the endoplasmic reticulum, or the membrane potential. Liberation of Ca^{2+} from intracellular stores occurs through two large channel complexes, composed of four subunits. Both are Ca^{2+}-induced Ca^{2+}-release channels (they need cytosolic Ca^{2+} in order to release Ca^{2+} from the store), but have distinct molecular weights and allosteric regulation mechanisms. The first is defined as the IP_3-receptor (ITPR), responding to the second messenger inositol-1,4,5-trisphosphate, the other the Ryanodine receptor (RYR), because it was shown to be sensitive to a plant alkaloid named Ryanodine (summarized in Figure 6-1).

Ca^{2+}-BINDING PROTEINS

EF-hand containing proteins

Of the Ca^{2+}-binding domains, the EF-hand is most frequently employed. It is composed of a helix-E, a metal-coordinating turn, and a helix-F in an arrangement that resembles the index finger and thumb (Figure 6-3). The EF-hand motif comprises roughly 29 amino acids, with a metal coordination loop composed of 12 amino acids. Most proteins contain two or four EF hands (EF domain) of which the affinity may vary between the different coordination sites. The affinity for Ca^{2+} varies enormously between EF hands, with K_D's ranging from 10 nM (parvalbumin) to 100 μM (some S100 proteins). Intracellular free Ca^{2+} concentrations range from 50 nM (lower limit of "resting" concentration) to 500 μM (in synaptic vesicles) and these are within the range of EF-hand affinities. The EF-hand motifs are flexible and are able to bind much larger metals such as Pb^{2+}, Ba^{2+}, and Sr^{2+} and they play a role in the toxicity of heavy metals (Kuma et al., 2012). The EF hand was first described in parvalbumin, a small protein extracted from skeletal muscle. Members of this family of small Ca^{2+}-binding proteins (12 kDa) play a role in muscle relaxation by removing free Ca^{2+} from troponin C and shuttling it to the Ca^{2+} pumps in the sarcoplasmic reticulum (Arif, 2009). Calmodulins (calcium modulated protein) are EF hand containing Ca^{2+} sensors that play crucial roles in signaling pathways and have been extensively studied in the context of signal transduction. Calcium ions change their conformation and the Ca^{2+}/calmodulin switch controls numerous effectors such as protein kinases, phosphatases, adenylyl cyclases, nucleotide phosphodiesterases, nitrogen oxide synthase, and ion channels. Calmodulins also bind components of the actin cytoskeleton machinery. A number of examples of the nearly 100 calmodulin effectors are shown in Figure 6-6). Among the EF-hand-containing proteins is a large family of S100 proteins, some of which bind Zn^{2+} and Cu^{2+}. They play roles in the control of toxic metal ions and numerous cellular activities as witnessed by the findings that abnormal S100-gene expression is detected in cardiomyopathy, Alzheimer, psoriasis, chronic inflammation, and tumor progression (Heizmann et al., 2007). Among the EF-hand-containing proteins, we also cite calcineurin B, a protein complexed with the calcineurin phosphatase (PPP3CB) and which plays a role in lymphocyte activation (dealt with in chapter 15 "Activating the Adaptive Immune System"). Recoverin (RCVRN) and GUCA1 are members of the neuronal calcium sensor proteins which form a distinct group in the EF-hand superfamily. They are expressed in the brain and retina and typically are formed by two pairs of EF-hand domains creating two globular structures (EF1/2 and EF3/4) connected by a short flexible hinge region. As we have discussed in Chapter 5 "Sensory Signal Processing" (Figures 5-6 and 5-12) they play an essential role in visual transduction. Other members of this branch are KCNIP3 (both a regulator of voltage-gated K^+ channels and a transcriptional repressor inhibited by Ca^{2+} and stimulated by Mg^{2+}), NCS1 (regulates conductivity of K^+ channels (KCND2)) and KCNIP1, -2 (regulate conductivity of voltage-gated K^+ channels (KCND3) in brain and cardiac cells) (Ames et al., 2012).

C2-domain containing proteins

The C2-domain occurs in protein kinase C, phospholipase-A and -C, phosphoinositide 3-kinaseC2, and in synaptotagmins. It was first recognized as the second conserved domain of protein kinase C, which is where the term "C2" derives from (Coussens et al., 1986). The number of coordination sites per Ca^{2+} is rather low (three or four) and tight binding requires the presence of other ligands with a predilection for the acidic phospholipids, knowingly phosphatidylinositol, phosphatidylserine, and phosphatidic acid. As a consequence, C2 domains bind Ca^{2+} at the interface between cytoplasm and phospholipid membranes which means that Ca^{2+} effectively acts as a membrane-recruiting agent for C2-carrying proteins.

Annexins

Annexins forms yet another family of Ca^{2+}-binding proteins, comprising 12 members (ANXA1-11, 13). These proteins carry four, and in one occasion six (ANXA6), annexin repeats (61 amino acids each). Like the C2 domain, the coordination of Ca^{2+} is insufficient (only five coordination sites are provided) and Ca^{2+} binding also requires the presence of acidic phospholipids (Weng et al., 1993). This property links annexins to many membrane-related events, such as the regulated organization of membrane domains and/or membrane–cytoskeleton linkages, certain exocytic and endocytic transport steps, and the regulation of ion fluxes across membranes (Gerke et al., 2005). Annexins came forth out of two parallel lines of research. The founding member of annexins is synexin, now named annexin 7. It was discovered during a search for proteins that promote fusion of secretion vesicles with the plasma membrane in the chromaffin cells of the adrenal medulla (so-called chromaffin granules) (Creutz et al., 1978). Indeed, annexins do play a role in establishing the contact between two membranes (but not in the fusion process). The second line of research concerns the identification of lipocortin, now named annexin 1 (ANXA1). Annexin 1 is a potent inhibitor of the soluble form of phospholipase A2 and has been extensively studied because its expression is induced by synthetic anti-inflammatory steroids (glucocorticoids). These are mainstay medicaments in the treatment of inflammatory disorders such as asthma, rheumatoid arthritis, and eczema (Flower and Blackwell, 1979; Wallner et al., 1986; D'Acquisto et al., 2008). Phospholipase A2 plays a key role in the initiation of production of the so-called eicosanoids (prostaglandins and leukotrienes) which are potent inflammatory mediators (return to Chapter 2 "An Introduction to Signal Transduction," section "Inflammatory mediators and vasoactive agents"). Later studies have shown that annexin one acts beyond phospholipase A2 and also prevents induction of inducible nitric oxide (iNOS), neutrophil migration, and facilitates apoptosis of white blood cells (Parente and Solito, 2004). As a consequence of its action, fewer white blood cells enter the tissue affected by the inflammatory disorder, thereby limiting their destructive action.

Glucocorticoids are a class of steroid hormones that bind to the glucocorticoid receptor (GR), which is present in almost every vertebrate animal cell. The name glucocorticoid (glucose + cortex + steroid) derives from its role in the regulation of the metabolism of glucose, its synthesis in the adrenal cortex, and its steroidal (cholesterol-based) composition. A less common synonym is glucocorticosteroid. Hydrocortisone, or cortisol, is the best known natural glucocorticoid. Examples of potent agonists that are used in the treatment of immune disorders are prednisolone, betamethasone, and hexamethasone.

Importantly, several annexins, including ANXA1, -A2, -A5, and -A6, display specific and distinct abilities to interact and promote membrane targeting of different isozymes of protein kinase C (PRKC) and promote targeting of components of the RAF-MEK-ERK pathway to early endosomes (Tebar et al., 2014; Hoque et al., 2014). They contribute to the assembly of novel signaling complexes after removal of receptors from the plasma membrane. The procedure resembles what we have described for arrestin-mediated signaling in response to adrenaline in chapter 3 "Regulation of Muscle Contraction by Adrenoceptors" (Figure 3-24). We leave the annexins with the mention that annexin 5 plays a role in membrane repair by binding to torn-membrane edges and by forming a Ca^{2+}-induced lattice that promotes membrane resealing (Bouter et al., 2011).

Ca^{2+}-buffering proteins

A number of Ca^{2+}-binding proteins, which carry numerous low affinity Ca^{2+}-binding sites ($K_D \sim 2\,mM$), reside in the endoplasmic reticulum and in mitochondria where they play a role in the buffering of Ca^{2+}. Among these are the glycoprotein-chaperones calreticulin (CALR) and calnexin (CANX). Calreticulin offers 25 binding sites and qualifies as a major Ca^{2+}-binding protein (Corbett and Michalak, 2000). Minor Ca^{2+}-binding proteins are the chaperones Grp78 (HSPA5) and Grp94 (HSP90B1). The functions of these chaperones and the formation of folding complexes which client proteins are influenced by Ca^{2+} (Coe and Michalak, 2009).

Skeletal and cardiac muscles express an additional Ca^{2+} binder in the endoplasmic reticulum, calsequestrin (CASQ), which has a much higher capacity, in the order of 70 Ca^{2+} ions per protein. In skeletal muscle, it was shown that calsequestrin not only acts as a readily accessible reservoir of Ca^{2+}, but it also enhances the Ca^{2+} influx through the voltage-dependent type Cav1.1 channel (CACNA1S) located in the plasma membrane. It connects with the channel through the transmembrane protein JP45 (Pertill et al., 2010; Mosca et al., 2013).

Ca^{2+} pumps, exchangers, and ion channels

Ca^{2+} pumps, Ca^{2+}/Na^{+} exchangers, and even voltage-sensitive ion channels carry Ca^{2+}-binding sites. In the case of the pumps and exchangers, it is the binding of Ca^{2+} that triggers transport activity (Hilge et al., 2006; Toyoshima, 2009). Concerning voltage-sensitive ion channels, Ca^{2+} binding facilitates ion permeability, although it may have another quite unexpected consequence. Recent studies showed that Ca^{2+} binding to the voltage-gated Ca^{2+} channel CACNA1A (P/Q-type, CaV2.1) directly stimulates fusion of vesicles (loaded with for instance neurotransmitters) with the plasma membrane through an interaction with synaptotagmin (a C2-domain containing Ca^{2+}-binding protein) (Bachnoff et al., 2013; Atlas, 2013).

Gelsolin-repeat containing proteins

The last proteins in the long list are adseverin (SCIN) and gelsolin (GSN). Both contain six gelsolin repeats. Adseverin is presumed to have a regulatory function in exocytosis by affecting the organization of the microfilament network underneath the plasma membrane (cortical actin). Gelsolin is a calcium-regulated actin-binding protein, playing a role of filament assembly, stabilization, and disassembly. When it binds the barbed (+) ends of actin filaments it acts as a capping protein, preventing depolymerization;

when binding at the pointed end (–) end, it promotes nucleation; and when binding in between the extremities it serves as a severing protein, breaking up existing filaments. It not only occurs in the cytosol but also in mitochondria, where it plays a role in the regulation of apoptosis (Burtnick et al., 2004). One of the gene splice variants, the one that gives rise to the inclusion of a signal peptide, is a secreted protein. It has obtained considerable interest when it was shown that an autosomal dominant D187N or D187Y mutation is linked with familial amyloidosis of Finnish type (FAF) also known as gelsolin amyloidosis (Solomon et al., 2012)

Finally, Ca^{2+}-binding domains occur in proteins with otherwise quite different domain architectures (Figure 6-4). It should be noted that when recognized by their amino acid sequence, EF, ANX, and C2 domains are not always functional Ca^{2+} binders.

Ca^{2+} RECEPTORS

The extracellular calcium concentration is controlled by the parathyroid gland, the kidneys, intestine, and bones, by means of parathyroid hormone (PTH), calcitonin, and 1,25$(OH)_2$ vitamin D. Together, they regulate calcium ion flux through the body compartments. The calcium-sensing receptor (CASR) was first identified in the bovine parathyroid gland where it regulates expression of parathyroid hormone. An increase in plasma-calcium concentrations activates the receptor leading to repression of secretion of the hormone. Loss of parathyroid hormone causes a reduction of osteoclast activity in the bone; it reduces Ca^{2+} reuptake in the nephrons; and it reduces Ca^{2+} sequestration in the digestive tract (through a reduction in expression of the Ca^{2+}-binding proteins S100G and CALB1).

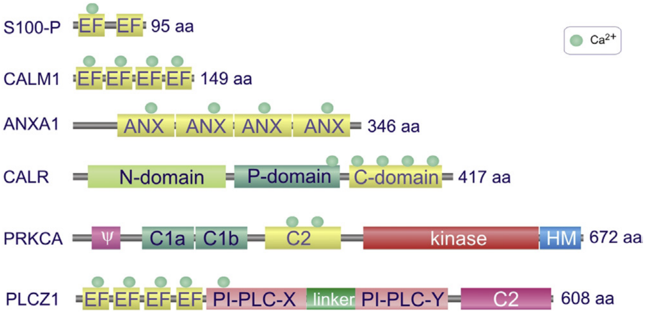


FIGURE 6-4 Some examples of domain architectures of Ca^{2+}-binding proteins. The recognition of a Ca^{2+}-binding domain through sequence analysis is not a guarantee for Ca^{2+} binding. Some domains/repeats have been mutated and fail to offer suitable coordination sites. In the case of the annexin repeat, one or more Ca^{2+}-binding sites are possible. The example shown in Figure 6-3 (structure from annexin 5) coordinates three Ca^{2+} ions.

In all, it prevents Ca^{2+} entering our body and it prevents its mobilization from the skeleton. The reverse process occurs when Ca^{2+} levels drop which gives rise to an increase in parathyroid hormone levels.

The Ca^{2+} receptor belongs to the type C superfamily of G protein-coupled receptors, together with two other Ca^{2+}-binding receptors, the metabotropic glutamate and γ-aminobutyric acid (GABA) type B receptor (refer to chapter 5, "Sensory Signal Processing," Figure 5-22). The calcium-sensing receptor forms a homodimer, held together by a disulphide bond in the N-terminal segment. Each receptor binds Ca^{2+} at two distinct locations; in the extracellular domain, where three sites have been identified, and in the transmembrane helical bundle only one site (Huang et al., 2007; Hu and Spiegel, 2007). It signals through Gαq or Gα11, both of which stimulate phospholipase C. Inactivating missense mutations in the receptor lead to hypercalcemia and neonatal severe hyperparathyroidism (MIM:239,200) (Pollak et al., 1993). Other tissues that express calcium-sensitive receptors are the kidneys, where the active receptor inhibits 1,25(OH)2 vitamin D, and bone, where almost all cell types express the receptor (Filopanti et al., 2013). For the two other types of Ca^{2+}-binding receptors, Ca^{2+} is not the primary ligand but acts as an allosteric ligand, in which glutamate or γ-aminobutyric acid contribute to the coordination of the ion.

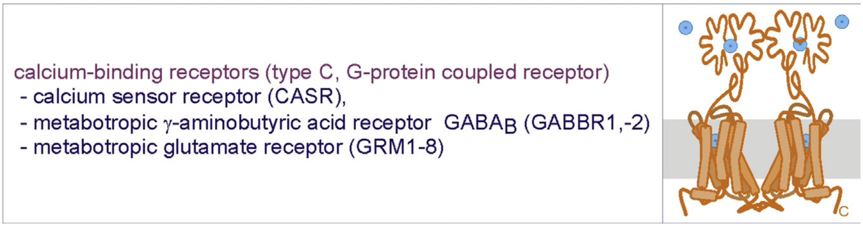
Parathyroid hormone, or its equivalent parathyroid-related protein, is highly expressed in thyroid carcinoma but also in breast, lung, and prostate cancers that have infiltrated the bone (metastasis). Osteoclasts play a role in the subsequent destructive process by releasing transforming growth factor-β, a growth factor that stimulates the production of parathyroid-related protein by the invading tumor cells (Grill et al., 1991; Johnson et al., 2011). This leads to excessive bone resorption by the osteoclasts and causes intractable pain and risk of fracture. Thus, it happened that during the second performance of Orfeo ed Euridice, composed by Gluck and conducted by Barbirolli in 1953 at the Royal Opera House in London, the contralto Ferrier, as a consequence of aggressive metastatic breast cancer, snapped her femur on stage and had to remain immobile throughout the last part of the opera with only few people in the audience noticing her distress. It was her last public performance (Ferrier, 2012).

Ca^{2+}/CALMODULIN-MEDIATED REGULATION OF PROTEIN ACTIVITY

Proteins that do not carry a Ca^{2+}-binding protein are not necessarily insensitive to Ca^{2+} because they may interact with Ca^{2+}-binding proteins.

Calmodulin is such an important intermediate; it senses calcium levels and relays signals to various effectors such as enzymes and ion channels. It is present in all eukaryotic cells and in the mammalian brain it even comprises about 1% of the total protein. Calmodulin, which is coded by three distinct bona fide genes *calm1*, *2*, and *3* (on chromosomes 14, 2, and 19, respectively) coding for absolutely identical proteins, is a small (only 17 kDa) dumbbell-shaped protein composed of two globular domains, each constituted of a pair of EF hands, tethered by a flexible linker (Figures 6-3 and 6-6). Its structure is similar to troponin C, the major difference being the length of the linker. Calmodulin came to light as a small activator subunit ("activating factor") of cyclic nucleotide phosphodiesterase obtained from bovine brain (Cheung, 1970; Kakiuchi and Yamazaki, 1970). Troponin C was discovered two years earlier, in 1968, in muscle tissue, and it can claim to be the first protein shown to be sensitive to calcium (Ebashi and Endo, 1968). Return to Chapter 3 "regulation of muscle contraction by adrenoceptors," Figure 3-19, for the role of troponin C in muscle contraction.

The flexible linker region allows calmodulin to interact with a wide range of proteins, estimated to be more than 100 (Yap et al., 2000). In the absence of Ca^{2+}, the helices pack so that their hydrophobic patches are not exposed. Binding of Ca^{2+} to the four EF hands induces a large conformational change, causing the terminal helices to expose hydrophobic surfaces. In this conformation, calmodulin typically wraps around its target with high affinity ($K_D \sim 10\,\text{nM}$), with the two globular domains gripping either side of it. A high content of methionine residues is believed to be responsible for target binding. Figure 6-6 illustrates the different conformations of "apo" calmodulin, Ca^{2+} bound and calmodulin wrapped around three targets: Ca^{2+}/calmodulin-dependent kinase Camk2a, myosin light-chain kinase Mylk2, and the intracellular domain of the K^+-channel Kcnn2. Calmodulin binding encourages the target sequence to adopt an α-helical arrangement so that it occupies the center of the hydrophobic tunnel. Depending on the target (and its calmodulin recognition mode), it displaces an auto-inhibitory domain, causes protein recruitment, causes protein dimerization or leads to altered protein domain interactions (Hoeflich and Ikura, 2002; Schumacher et al., 2001; Reichow et al., 2013). In Chapter 4, "Cholinergic Signaling and Muscle Contraction," we have



calcium-binding receptors (type C, G-protein coupled receptor)
- calcium sensor receptor (CASR),
- metabotropic γ-aminobutyric acid receptor $GABA_B$ (GABBR1,-2)
- metabotropic glutamate receptor (GRM1-8)

FIGURE 6-5 Ca^{2+}-binding receptors.

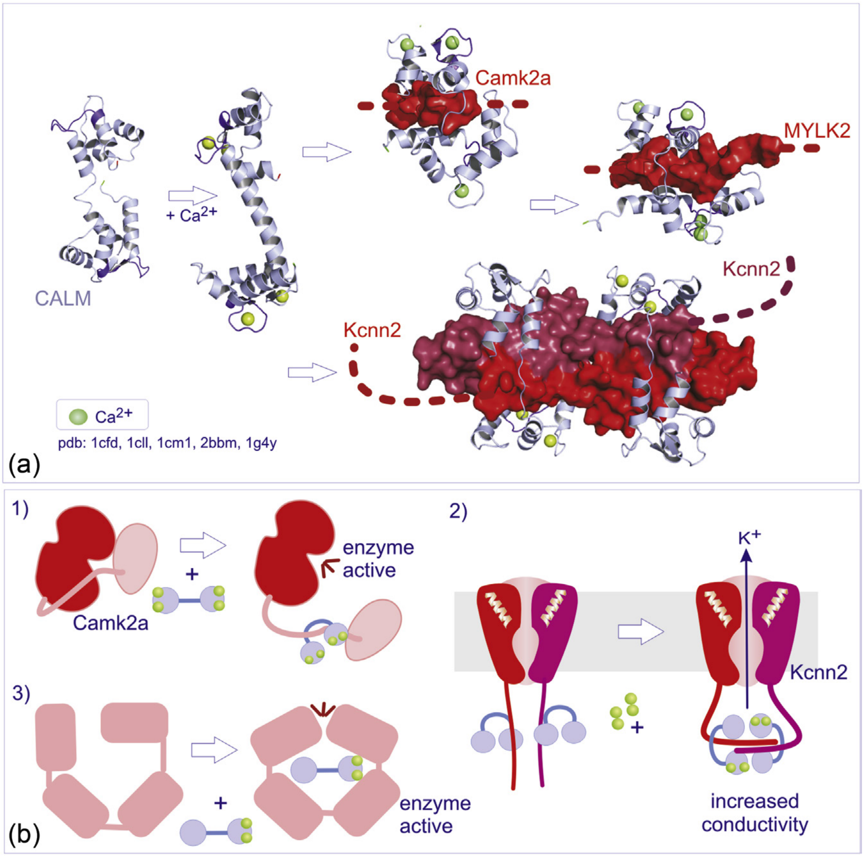
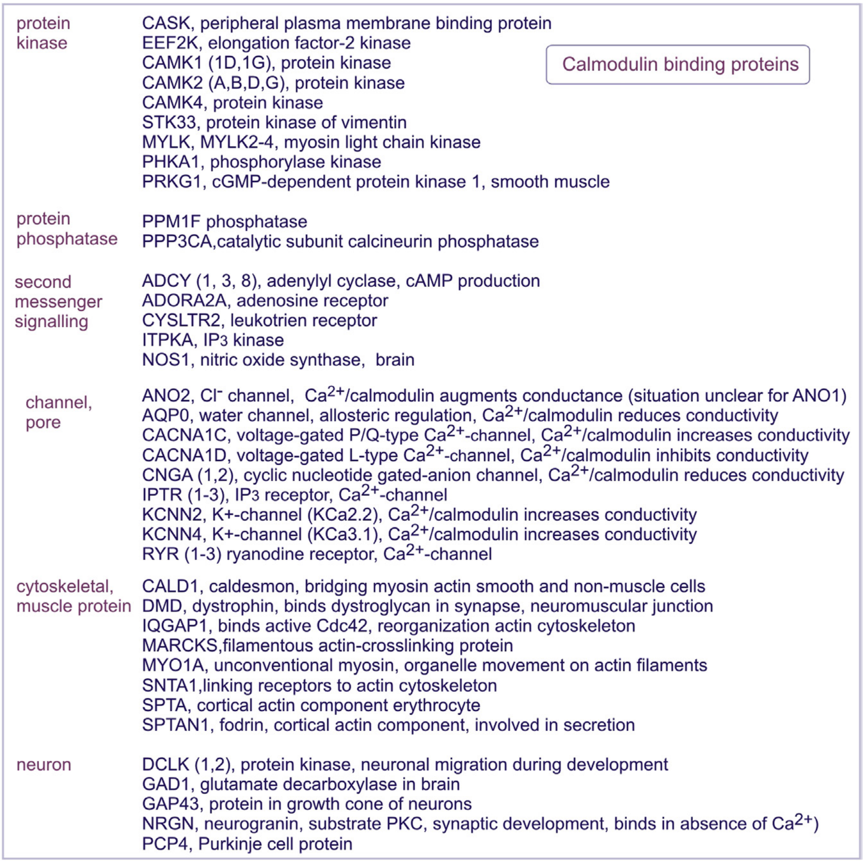


FIGURE 6-6 Structural changes in calmodulin and its binding to multiple effector proteins. (a) Calmodulin occurs in an inward folded state. Binding of Ca^{2+} at the four EF hands brings about an extended conformation, exposing a long α-helix linker region with two hydrophobic patches. This allows calmodulin to wrap around target proteins of different types, such as Ca^{2+}/calmodulin protein kinase (Camk2) and myosin light-chain kinase (MYLK2) thereby removing an auto-inhibitory constraint. Calmodulin also binds a Ca^{2+}-activated K^+ channel (Kcnn2) and here it causes dimerization of the intracellular domain which leads to increased channel conductivity. Pdb: 1cfd, 1cll, 1cm1, 2bbm, 1g4y. The coordinates of the dimer structure of Kcnn2 was kindly provided by Dr Maria Schumacher, Duke University School of Medicine, Durham, USA. (b) Protein alteration mechanisms caused by calmodulin upon Ca^{2+} binding. It either (1) removes an auto-inhibitory domain from the catalytic site (many protein kinases), or (2) causes domain dimerization (example of the K^+-channel Kcnn2), or (3) causes remodeling of the active site (example of NO synthase and anthrax edema factor).

shown how calmodulin wraps around a much more bulky target, NO synthase (NOS1). Return to Figure 4-22.

A list of calmodulin targets is shown in Figure 6-7. Calmodulin is a central regulatory element in gene regulation (for instance, through the activity of CAMK members), protein synthesis, fast axonal transport, smooth muscle



Calmodulin binding proteins

protein kinase	CASK, peripheral plasma membrane binding protein
	EEF2K, elongation factor-2 kinase
	CAMK1 (1D,1G), protein kinase
	CAMK2 (A,B,D,G), protein kinase
	CAMK4, protein kinase
	STK33, protein kinase of vimentin
	MYLK, MYLK2-4, myosin light chain kinase
	PHKA1, phosphorylase kinase
	PRKG1, cGMP-dependent protein kinase 1, smooth muscle
protein phosphatase	PPM1F phosphatase
	PPP3CA,catalytic subunit calcineurin phosphatase
second messenger signalling	ADCY (1, 3, 8), adenylyl cyclase, cAMP production
	ADORA2A, adenosine receptor
	CYSLTR2, leukotrien receptor
	ITPKA, IP_3 kinase
	NOS1, nitric oxide synthase, brain
channel, pore	ANO2, Cl^- channel, Ca^{2+}/calmodulin augments conductance (situation unclear for ANO1)
	AQP0, water channel, allosteric regulation, Ca^{2+}/calmodulin reduces conductivity
	CACNA1C, voltage-gated P/Q-type Ca^{2+}-channel, Ca^{2+}/calmodulin increases conductivity
	CACNA1D, voltage-gated L-type Ca^{2+}-channel, Ca^{2+}/calmodulin inhibits conductivity
	CNGA (1,2), cyclic nucleotide gated-anion channel, Ca^{2+}/calmodulin reduces conductivity
	IPTR (1-3), IP_3 receptor, Ca^{2+}-channel
	KCNN2, K+-channel (KCa2.2), Ca^{2+}/calmodulin increases conductivity
	KCNN4, K+-channel (KCa3.1), Ca^{2+}/calmodulin increases conductivity
	RYR (1-3) ryanodine receptor, Ca^{2+}-channel
cytoskeletal, muscle protein	CALD1, caldesmon, bridging myosin actin smooth and non-muscle cells
	DMD, dystrophin, binds dystroglycan in synapse, neuromuscular junction
	IQGAP1, binds active Cdc42, reorganization actin cytoskeleton
	MARCKS,filamentous actin-crosslinking protein
	MYO1A, unconventional myosin, organelle movement on actin filaments
	SNTA1,linking receptors to actin cytoskeleton
	SPTA, cortical actin component erythrocyte
	SPTAN1, fodrin, cortical actin component, involved in secretion
neuron	DCLK (1,2), protein kinase, neuronal migration during development
	GAD1, glutamate decarboxylase in brain
	GAP43, protein in growth cone of neurons
	NRGN, neurogranin, substrate PKC, synaptic development, binds in absence of Ca^{2+})
	PCP4, Purkinje cell protein

FIGURE 6-7 Some calmodulin-binding proteins and the cellular processes they are involved in. The categories shown on the left (protein kinase, phosphatase, etc.) are arbitrary; they are not formal and merely aim to illustrate the diversity of protein targets.

contraction, secretion, growth-cone elongation, organelle shaping, ion-channel function, and cell motility. An unusual characteristic of calmodulin-binding proteins is their lack of a conserved amino-acid sequence and this makes it difficult to extract information from protein databases. Moreover, not all targets require the calcium ion for calmodulin binding and some interactions are even inhibited by calcium. Target interactions can therefore be categorized as Ca^{2+} dependent, Ca^{2+} independent, and Ca^{2+} inhibited (O'Day, 2003). The list of calmodulin-binding proteins shown in Figure 6-7 serves merely to illustrate the variety of targets and the different cellular processes they are involved in. In conclusion, Ca^{2+} is everywhere and so is calmodulin. We will return to the action of calmodulin when describing its regulation of Ca^{2+}/calmodulin-dependent kinase.

Bacillus anthracis, a spore-forming bacterium, is the causative agent of anthrax. Virulent strains secrete two toxins, lethal factor (LF) and edema factor (EF). EF is a latent, soluble, adenylyl cyclase. Binding of Ca^{2+}/calmodulin causes a massive conversion of ATP into cAMP. Although the extent of this effect varies according to cell type, cellular cAMP levels may reach a 1000-fold increase, giving rise to a 30% reduction in the intracellular ATP level (which normally is in the order of 5–10 mM). Of note, edema is not only caused through an increase in vascular permeability, but also through an indirect mechanism involving inflammatory mediators. Calmodulin is a well-chosen cofactor because it is both abundantly present and, with respect to the amino-acid sequence, completely invariant among all vertebrates.

Web resource

The cellular calcium information server at the Ontario Cancer Institute. Among the services is the search option for a putative calmodulin binding site http://calcium.uhnres.utoronto.ca/.

TOOLS TO STUDY THE ROLE OF Ca^{2+} IN CELLULAR PROCESSES

In the next section, we will discuss the different tools that have been developed to study the involvement of Ca^{2+} in cell signaling. Like for the second messenger cAMP, research increasingly focuses on spatiotemporal characteristics of elevated Ca^{2+} levels, rather than global changes.

Sensing changes in intracellular Ca^{2+} concentration

To detect and follow the progress of changes in [Ca^{2+}] in living cells has required the introduction of a Ca^{2+}-sensing agent. This presented a formidable challenge. It was first attempted in 1928, by injecting the dye alizarin sulfonate into an ameba. Apparent evidence of a local increase in Ca^{2+} was obtained when a visible precipitate formed at the site of pseudopod formation (Pollack, 1928). Unfortunately, it later emerged that alizarin is not specific for Ca^{2+}. More unfortunately, the development of practical Ca^{2+} indicators suitable for use with mammalian cells took a further 50 years. In the meantime, intracellular calcium levels were mostly investigated by measuring transmembrane fluxes of the radioisotope ^{45}Ca. It is possible to measure

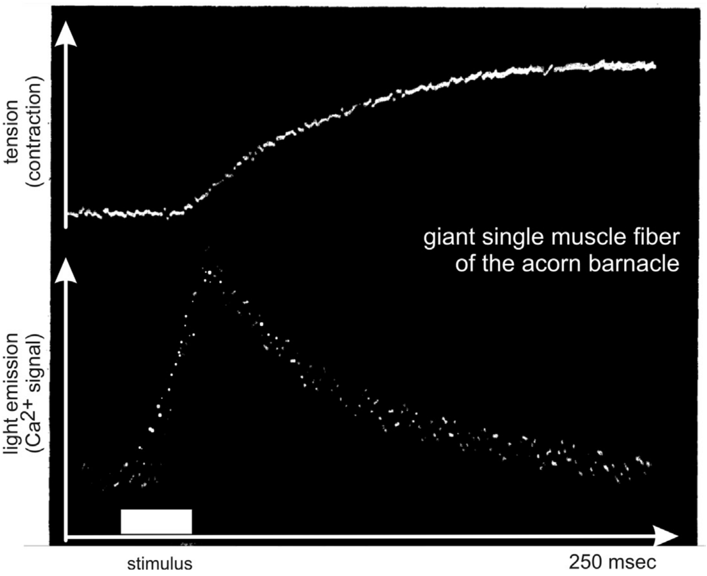


FIGURE 6-8 First detection of a Ca^{2+} transient in the giant muscle fiber of the acorn barnacle injected with aequorin. The isolated muscle (cross-striated type) was made to contract through electrical stimulation (lasting 20 ms) applied to one of the nerves at the muscle margins, where the fiber attaches to the scutes. Tension development and Ca^{2+}-mediated fluorescence were measured simultaneously. The slightly modified version was obtained with permission from Ridgeway and Ashley (1967).

changes in *total* cell [Ca^{2+}] by this means, but impossible to determine how concentrations have changed within particular intracellular compartments, such as in the cytosol.

A better alternative was provided by jelly fish from which the Ca^{2+}-activated light-emitting protein aequorin can be extracted (Shimomura and Johnson, 1969). Its molecular mass is 22 kDa and it cannot cross membranes, so prior to the era of transfection techniques, its use was limited to larger cell types into which it could be microinjected. Once in the cytosol, an increase in free Ca^{2+} concentration causes the protein to release a pulse of light. In important early work, aequorin was used with preparations such as giant single muscle fiber of the acorn barnacle *Balanus nubilus* (Figure 6-8). This muscle fiber provided not only the first direct observations of a so-called "Ca^{2+} transient," a biphasic rise and fall of cytosol Ca^{2+}, but also its intimate connection with muscle contraction (refer to Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," section "Muscle contraction: striated versus smooth muscle") (Ridgeway and Ashley, 1967; Ashley, 1969). Later studies with squid giant axon and aequorin revealed how calcium transients, occurring in the presynaptic terminal upon membrane depolarization (action potential), play a role in neurotransmitter release (Llinas et al., 1972).

Ca^{2+}-sensitive photoproteins, extracted in their active form from marine invertebrates such as the cnidarians, *Aequorea* (Shimomura et al., 1962), and Obelia (Campbell, 1974), undergo an internal molecular rearrangement activated by Ca^{2+}. The conformational change oxidizes a luminophoric prosthetic group, coelenterazine, emitting a pulse of blue light.

The measurement of free intracellular Ca^{2+} became a practical reality with the design by Roger Tsien of the first of a series of synthetic Ca^{2+} indicators. The fluorescence of these compounds is sensitive to Ca^{2+} concentration in the range 50 nM to 1 μM (Tsien, 1980; Tsien et al., 1982; Grynkieuwicz et al., 1985). The first of these to be developed was called Quin2 and was soon followed by an improved indicator, Fura2 (Figure 6-9). They are effectively Ca^{2+} chelators, like EGTA (Figure 6-2), with not only high affinity for Ca^{2+} and low affinity for Mg^{2+}, but coupled to a fluorophore and an acetoxymethyl ester derivative also to allow them to penetrate the cell membrane (Tsien, 1981). Upon entry in the cytosol, the endogenous esterase activity releases the acidic form of the indicator. These Ca^{2+} indicators exhibit progressive wavelength shifts in their fluorescence excitation spectrum as Ca^{2+} is bound—an example is shown in Figure 6-9(b).

Collective versus individual cell measurements

Initially, measurements were performed on suspensions of cells undergoing treatment with a soluble ligand in a fluorometer cuvette. In this situation, the recorded emission represents the sum of contributions from each cell in the light beam and revealing characteristic transient response patterns as illustrated in Figure 6-10. Importantly, these early studies revealed that in many cell types the initial Ca^{2+} peak is not affected when Ca^{2+} is removed from the external solution (by adding an excess of EGTA). Its source therefore must be Ca^{2+} ions mobilized from internal stores. The second phase of the signal, on the other hand, is eliminated by the effective removal of extracellular Ca^{2+} (Figures 6-10(a)) (Merrit and Rink, 1987).

When Ca^{2+} indicator dyes are used with single cells observed under a fluorescence microscope, the time courses are very different. Following treatment, the signals may appear as trains of sharp spikes or as a series of smooth oscillations (Figure 6-10(b–d)). The periodicity, frequency, and amplitude tend to vary with the concentration of the stimulus (Jacob et al., 1988). Going a step further, and observing Ca^{2+} movements under the confocal fluorescence microscope revealed that Ca^{2+} diffuses in waves across the cell. The intensity maps are obtained

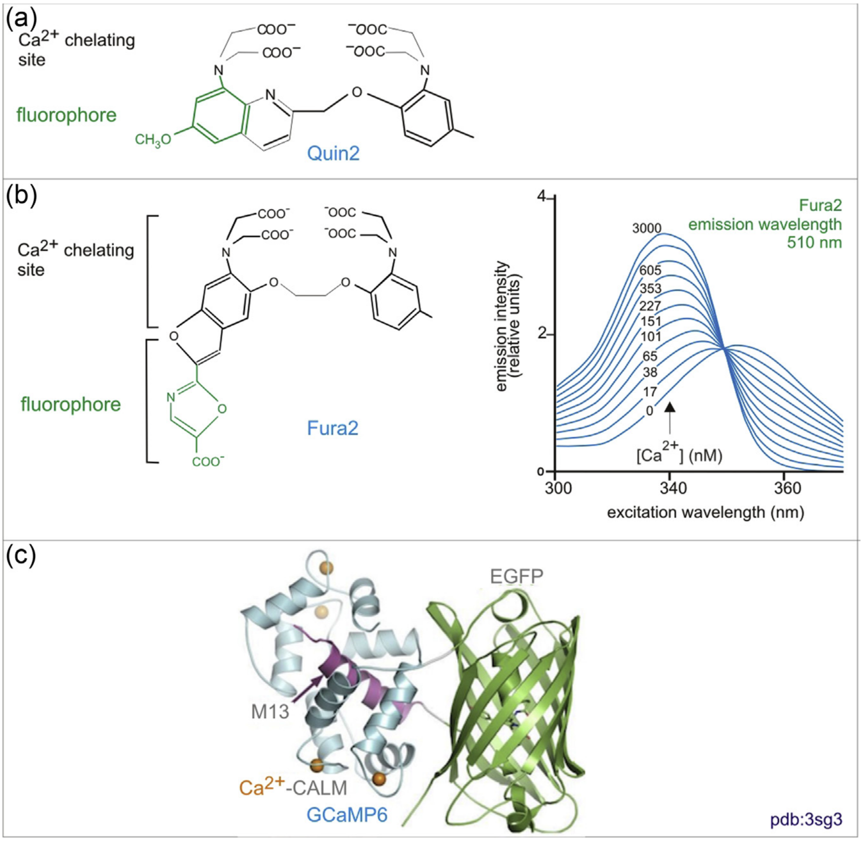


FIGURE 6-9 **Molecular structure of Quin2 and Fura2 and the fluorescence excitation spectrum of Fura2.** (a) Molecular structure of Quin2. (b) Molecular structure of Fura2 and its excitation spectrum at increasing Ca^{2+} concentrations. Excitation is provided at 340 nm (near UV) and the intensity of emission is measured at 510 nm (green fluorescence). (c) The genetically encoded Ca^{2+} indicator GCaMP3 is composed of three parts: a calcium-binding moiety, calmodulin, a linker which interacts with calmodulin, M13, and a green fluorescent protein. The green light emission intensifies when Ca^{2+} binds calmodulin.

by using a pseudocolor display in which "warmer" colors (orange and red) indicate higher Ca^{2+} levels. Possible explanations of these phenomena are discussed later in this chapter.

A confocal microscope is able to generate optical sections of cells and small lumps of tissues. The resolution and quality are higher than those of a standard fluorescence microscope. Moreover, a set of such two-dimensional images, obtained by moving the plane of focus successively through the specimen, can provide a three-dimensional image of cell fluorescence.

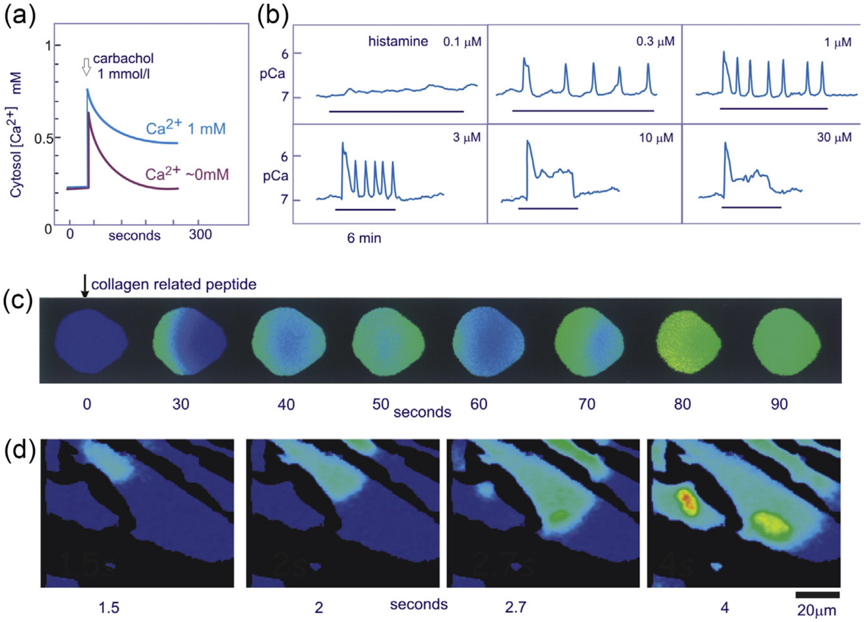


FIGURE 6-10 **Changes in cytosolic-free Ca^{2+} as measured under different experimental conditions.** (a) The trace shows elevation of Ca^{2+} in parotid acinar-cell suspension measured by Fura2. The stimulus (carbachol) was applied (at the time indicated by the arrow) to cells in the presence of extracellular Ca^{2+} (blue line) or near absence of Ca^{2+} (violet). *Trace adapted from Merritt and Rink (1987).* (b) The effect of different concentrations of histamine on intracellular [Ca^{2+}] in a single human endothelial cells. The bars indicate the duration of the application of histamine. The Ca^{2+} indicator was Fura2. Note that an increase of histamine has little effect on the amplitude, but markedly increases the frequency of Ca^{2+} spikes, which at very high concentrations fuse into an apparent constant elevated level. *Adapted from Jacob et al. (1988).* (c) Ca^{2+} waves in a megakaryocyte, as observed under the confocal microscope, in response to the addition of a collagen-related peptide (CRP). Note a successive slow wave across the cell. The initial waves are followed by a sustained increase lasting up to 5 min (not shown). The cell is approximately 40 µm in diameter. *Image from Mountford et al. (1999).* (d) Ca^{2+} waves in single astrocytes, as observed under the confocal microscope, in response to ATP (which activates purinergic receptors). A slow wave traverses the cells, progressively increasing its amplitude (yellow/red area). *From Michael Duchen (Boitier et al., 1999).*

To enhance resolution of subcellular Ca^{2+} events even further Ca^{2+} sensors have been developed that can bind genetically modified (recombinant) proteins and these allow nanoscale measurements. For more information, we suggest the following articles:

- Rizzuto. R., Simpson, A.W., Brini. M., Pozzan. T., 1992. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aeqorin. Nature 358, 325–327.
- Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.A., Ikura, M., Tsien, R.Y., 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature 388, 882–887.

- Nagai, T., Sawano, A., Park, E.S., Miyawaki, A., 2001. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. Proc. Natl. Acad. Sci. U.S.A 98, 3197–3202.
- Griffin, B.A., Adams, S.R., Tsien, R.Y., 1998. Specific covalent labeling off recombinant protein molecules inside live cells. Science 281, 269–272.
- Tour, O., Adams, S.R., Kerr, R.A., Meijer, R.M., Sejnoswki, T.J., Tsien, R.W., Tsien, R.Y., 2007. Calcium green FIAsH as a genetically targeted small-molecule calcium indicator. Nat. Chem. Biol. 3, 423–431.

Roger Tsien who engineered the fluorescent Ca^{2+} indicators also contributed to the development of Ca^{2+}-sensitive fluorescent proteins and shared the Nobel Prize in chemistry of 2008 with Osamu Shimomura and Martin Chalfie for "the discovery and development of the green fluorescent protein, GFP."

Green fluorescent protein is a 20 kDa protein found in the same jellyfish *Aequoria victoria* that is the source of aequorin. Mammalian cells may be transfected to express GFP alone or as a fusion partner to another protein, rendering it fluorescent and allowing its location to be tracked in living cells. Engineered forms of GFP have been developed that offer improved fluorescence and emit other colors such as enhanced GFP (EGFP), Cyan (CFP), yellow (YFP), and enhanced YFP (EYFP).

Delivering calcium indicators by genetically encoded Ca^{2+} sensors

Sensitive synthetic indicators are delivered by invasive chemical or physical methods that are not suitable for whole animal or selective cell studies (in a heterogeneous population). Genetically encoded sensors on the contrary can be targeted to specific cell types for noninvasive imaging of identified neurons and neuronal compartments. Moreover, they may be functional over long periods of time (months). The gene can be delivered by incorporating the DNA into a viral vector such as the adeno-associated virus (AAV), through classic plasmid transfection techniques or through transfection into an embryonic stem (ES) cell line followed by the creation of transgenic mice (Zariwala et al., 2012). A genetically encoded Ca^{2+} indicator (GECI) with high sensitivity is GCaMP6 (Figure 6-9(d)) (Chen et al., 2013). It is composed of calmodulin connected to the calmodulin-binding sequence (M13) of myosin-light chain kinase and attached to green fluorescent protein. When exposed to light in the blue to ultraviolet range the chimeric protein exhibits bright green fluorescence which is greatly enhanced when Ca^{2+} is bound to the calmodulin moiety (Nakai et al., 2001). When the protein is extended with a C-terminal CAAX box, the cell will add a fatty acid and/or an isoprenoid chain thus converting the protein into an integral membrane indicator (particularly suitable for detecting membrane-channel mediated Ca^{2+} entry (Tsai et al., 2014)). Using this indicator, action potentials can be monitored not only in multiple neurons but also in tiny synaptic compartments over multiple imaging sessions separated by months and omit the usage of electrodes.

Using Ca^{2+} ionophores to impose a rise in Ca^{2+}

The Ca^{2+} ionophores are lipid-soluble, membrane-permeant ion carriers that are specific for Ca^{2+}. They were originally isolated from cultures of *Streptomyces* and include the antibiotics A23187 (524 Da) and ionomycin (709 Da). They may be used to convey Ca^{2+} ions into cells and during the 1970s they were applied to numerous preparations, particularly in the field of secretion. Among the observed effects were responses similar to those that follow biological stimulation, but without the involvement of receptor-associated mechanisms. This revealed a widespread involvement of Ca^{2+} in activation mechanisms, establishing it as a legitimate second messenger alongside cyclic AMP. However, ionophores are blunt weapons and although they certainly enable the introduction of Ca^{2+}, seemingly to induce biological responses, it proved hard to exert control over the concentrations achieved.

MECHANISMS THAT ELEVATE CYTOSOL Ca^{2+} CONCENTRATION

Ca^{2+}-mobilization between different compartments in excitable and non-excitable cells

We consider three Ca^{2+} compartments knowingly: the cell's environment, cell organelles (including the nucleus), and the cytosol. What follows is mainly about the regulation of cytosolic-free Ca^{2+}. With respect to Ca^{2+} signaling, we distinguish between two cell types: excitable and non-excitable. We define excitable cells as those that possess voltage-sensitive Na^+ channels (members of the SCN family) located in the plasma membrane and which, once a threshold membrane potential has been reached, cause rapid membrane depolarization. When the depolarization signal propagates from one cell to another we speak of an action potential. Typical excitable cells are neurons and their derivatives such as chromaffin cells (expressing one the following sodium channel, SCN1-3A, 5A, 7-11A), striated muscle (expressing SCN4A in skeletal, SCN5A in heart), and gastrointestinal smooth muscle cells (expressing mainly SCN5A) (Beyder, 2012). Non-excitable cells normally lack these channels or they are not expressed in the plasma membrane.

In excitable cells, the rise in intracellular-free Ca^{2+} can be initiated by membrane depolarization, causing the very fast entry of extracellular Ca^{2+}

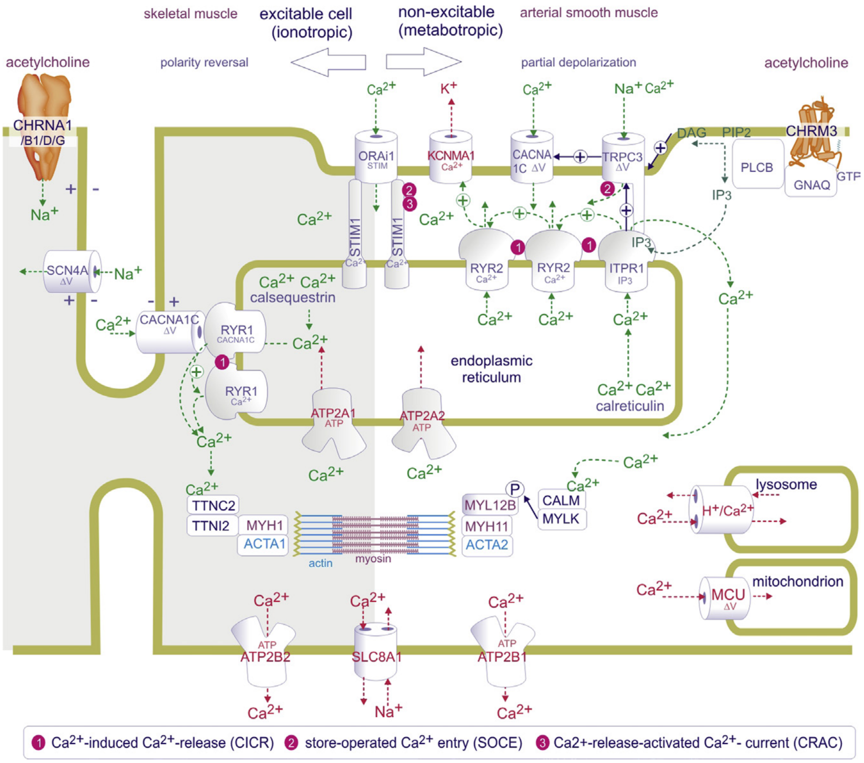


FIGURE 6-11 Mechanisms that control the concentration of cytosolic-free Ca^{2+} in striated (left, gray background) and smooth muscle (right). The events are described in the text. It should be noted that ITPR and RYR are not necessary in the same subcellular compartment in smooth muscle (Flynn et al., 2001).

through voltage-sensitive Ca^{2+} channels (ionic transmission mode), whereas in non-excitable cells this is necessarily caused, in a more delayed fashion, by receptor-stimulated production of second messenger such as inositol 1,4,5-trisphosphate (IP_3) acting on intracellular Ca^{2+}stores (Michell, 1975; Berridge and Irvine, 1984) or cyclic nucleotides acting on non-selective Ca^{2+}-permeant channels (metabotropic transmission mode) (Figure 6-11). However, both excitable and non-excitable cells require Ca^{2+} channels operating in both the plasma membrane and in cell organelles for the propagation of the initial Ca^{2+} signal. Both cell types employ similar modes of signal termination, which involves Ca^{2+} pumps and exchangers located both in the plasma membrane and in cell organelles (return to Figure 6-1 for a graphic summary). Finally, as we have shown for the sensory cells in the retina and olfactory epithelium, second-messenger-mediated signals can be translated in voltage-dependent Ca^{2+} currents (employing CACNA1F) at the synaptic junction (return to Chapter 5, Figure 5-7).

Ca^{2+} signaling mechanisms already discussed in previous chapters

- Chapter 3 "Regulation of Muscle Contraction by Adrenoceptors," where we discussed signaling in the heart and in arterial smooth muscle, summarized in Figure 3-22 and in Figure 3-28, respectively. To learn about phospholipase C and the production of inositol-1,4,5-trisphosphate (IP_3) return to Figures 3-15 to 3-18 and their accompanying text sections.
- Chapter 4 "Cholinergic Signaling and Muscle Contraction," where the role of Ca^{2+} is discussed in the context of contraction of skeletal muscle, summarized in Figure 4-12, bronchial smooth muscle summarized in Figure 4-19, and in bronchial epithelial mucus secretion summarized in Figure 4-20.
- Chapter 5, "Sensory Signal Processing" where we discussed the control of Ca^{2+} entry through cyclic nucleotides acting on CNGA1 in the retina and CNGA2 in olfactory epithelium, summarized in Figures 5-09 and 5-17, respectively.

Non-excitable cells have been reported to express particular splice variants of two voltage-gated Na^+ channels, SNC5A and SNC8A, but they are not at the plasma membrane. They are detected in endosomes and may play a role in migration (Carrithers et al., 2009). Intracellular expression of these channels correlates with invasiveness of transformed cells (Fraser et al., 2003).

Description of the Ca^{2+} mobilization process in skeletal and smooth muscle

Figure 6-11 depicts a generic scheme of a number of proteins involved in the regulation of intracellular Ca^{2+} with skeletal (shaded area) and atrial smooth (white area) muscle as examples. For skeletal muscle, complete reversal of membrane polarity, initiated by acetylcholine binding to the nicotinic receptor and subsequently enabled by opening of the voltage-gated Na^+ channel (SNC4A), causes a conformational change in the voltage-sensitive Ca^{2+} (CACNA1C) and this, without a necessary Ca^{2+} current, causes the opening of the RYR1 channel, allowing Ca^{2+} to enter the cytoplasm. Non-engaged RYR1 channel subsequently increase their conductivity through Ca^{2+}-induced Ca^{2+} release (CICR). Of note, in cardiac muscle, Ca^{2+} entry through CACNA1C is essential and this causes the opening of RYR2. Intracellular Ca^{2+} brings about muscle contraction by rendering actin accessible to myosin-1 (MYH1). The Ca^{2+} signal is necessarily brief because voltage-gated Na^+ channels rapidly become refractory after membrane depolarization. The subsequent return to the resting membrane potential will block Ca^{2+} permeability of the CACNA1C channel and, thus, arrests its entry (followed by the closure of RYR1).

For non-excitable smooth muscle cells, the process starts by acetylcholine-mediated activation of a G protein (GNAQ) and the conversion of phosphatidylinositol-4,5-bisphosphate by PLCβ into diacylglycerol and inositol-1,4,5-trisphosphate (IP_3). Binding of IP_3 to its receptor, ITPR1, reduces the sensitivity for Ca^{2+} inhibition and this leads to the release of Ca^{2+} from the endoplasmic reticulum. The signal is enhanced by a regenerative cascade of Ca^{2+}-induced Ca^{2+} release, whereby numerous RYR2 channels open. The open state of IPTR1 can also, through direct contact, increase the conductivity of TRPC3, a plasma membrane located in non-selective cation channel (Na^+, Ca^{2+}) (Mujica and González, 2011). The increasing presence of diacylglycerol in the plasma membrane further enhances conductivity of this channel (Hofmann et al., 1999). The influx of Na^+ and Ca^{2+} leads to a modest membrane depolarization, which causes an increase in conductivity of the voltage-sensitive Ca^{2+} channel CACNA1C, thus contributing to the increase in cytosolic free Ca^{2+}. Remember, contraction in smooth muscle is initiated by Ca^{2+}/calmodulin-mediated activation of myosin light-chain kinase (MYLK) which phosphorylates the small regulatory subunit (MYL12B) of smooth muscle

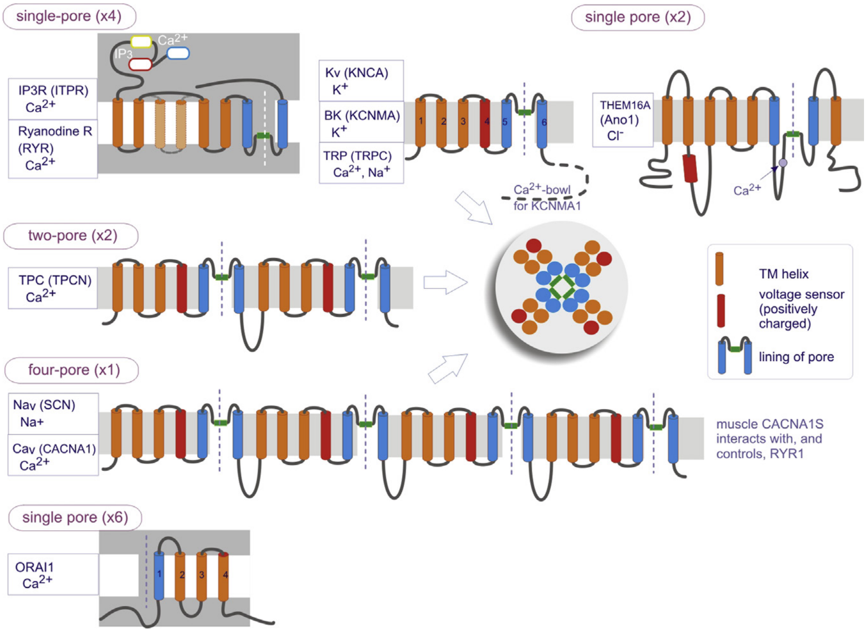


FIGURE 6-12 Membrane topology of Ca^{2+}-permeable channels, ion channels regulated by Ca^{2+}, and of ion channels implicated in the regulation of Ca^{2+} mobility (through their regulation of membrane potential). Note that with the exception of ITPR and ORAI, the channels all contain a voltage sensor (in red) and are thus in principle voltage gated. A subset of voltage-sensitive channels assembles in a typical pore-forming configuration, depicted in the circle. This helical arrangement may arise from folding of a monomer (four-pore channels), the assembly of dimers (two-pore channels), or tetramers (single-pore channels). Information about the topology of THEM16A from Terashima et al. (2013).

myosin (MYH11). This removes a constraint on the ATPase cycle and thus initiates the actin–myosin crossbridge cycle.

The extent of Ca^{2+} entry caused by TRCP3 may be constrained by the engagement of yet another ion channel, this time a Ca^{2+}-gated K^+ channel (KCNMA1). It contains a cytosolic "Ca^{2+} bowl" which, when occupied, increases channel conductivity (Figure 6-12) (Yuan et al., 2010). Enhanced K^+ conductivity requires μM concentrations of Ca^{2+}, a concentration which normally is not reached in the cytoplasm but may occur at discrete spots near the Ca^{2+}-entry sites, hence the importance of precise positioning of these channels (Narayanan et al., 2012; Hu and Zhang, 2012). The outflow of K^+ causes membrane hyperpolarization and this attenuates Ca^{2+} entry through CACNA1V and probably TRCP3. The KCNMA1 channel plays a role in the fine-tuning of blood pressure (Tomás et al., 2008).

Concerning the removal of cytosolic Ca^{2+}, excitable and non-excitable cells employ the same proteins but they not necessarily express the same gene variants. The endoplasmic reticulum harbors powerful Ca^{2+} pumps (so-called SERCA), which is ATP2A1 in skeletal muscle and ATP2A2 in arterial smooth muscle. Similar pumps are also located in the plasma membrane, this time ATP2B1 in skeletal muscle and ATP2B2 in arterial smooth muscle. Besides the pumps, the exchange protein SLC8A (an antiporter) located in the plasma membrane also expulses Ca^{2+} and is driven by the strong Na^+-electrochemical gradient. Lysosomes and mitochondria too participate in the removal of Ca^{2+}, employing an as yet poorly defined H^+/Ca^{2+} exchanger in the lysosome and a better-defined uniporter, MCU, in the inner membrane of the mitochondrion. Figure 6-12 illustrates the membrane topology of the different Ca^{2+}-permeant channels, of ion-channels that are regulated by Ca^{2+}, and of the channels that regulate Ca^{2+} mobility (through membrane potential).

Replenishing the intracellular Ca^{2+} stores

During each cycle of depolarization or production of IP_3, the cell loses some of its Ca^{2+}, expulsed from the cytosol through the outer membrane pumps and exchangers. In the case of smooth muscle, the transient Ca^{2+} entry through TRPC3 and CACNA1C channels apparently does not make up for this loss. Over time this would lead to depletion of the internal stores. Lowering Ca^{2+} levels are detected in the endoplasmic reticulum through the EF-hand of the STIM1 protein. In the presence of Ca^{2+} the cytosolic domain of this protein is folded unto itself. Ca^{2+} depletion leads to clustering and then to unfolding (Zhou et al., 2013). The lysine-rich N-terminal of the unfolded proteins is thought to bind the pore-forming helices of ORAI1 and this may be the mechanism by which they enhance conductivity (Zhou et al., 2013; Hou et al., 2012). STIM1-mediated opening of ORAI1 gives rise to a current that is referred to as the Ca^{2+} release-activated Ca^{2+} (CRAC) current, first observed in T-lymphocytes during the very first events in the process of clonal expansion (Kuno et al., 1986). The entering Ca^{2+} is shuttled into the endoplasmic reticulum by the Ca^{2+} pumps (members of the ATP2A family) (Figures 6-11 and 6-13). The human genome contains three ORAI and two STIM genes.

It is important to note that IP_3 is not the sole Ca^{2+}-mobilizing second messenger. Other messenger molecules that can release Ca^{2+} into the cytosol include the two nucleotides cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP), and the lipid sphingosine-1-phosphate. Arachidonic acid, produced upon Ca^{2+}-mediated activation of PLA2, causes Ca^{2+} entry through ORAI1/ORAI3.

Web resource

Kyoto Encyclopedia of genes and Genomes, KEGG, Calcium signaling pathway map ko04020

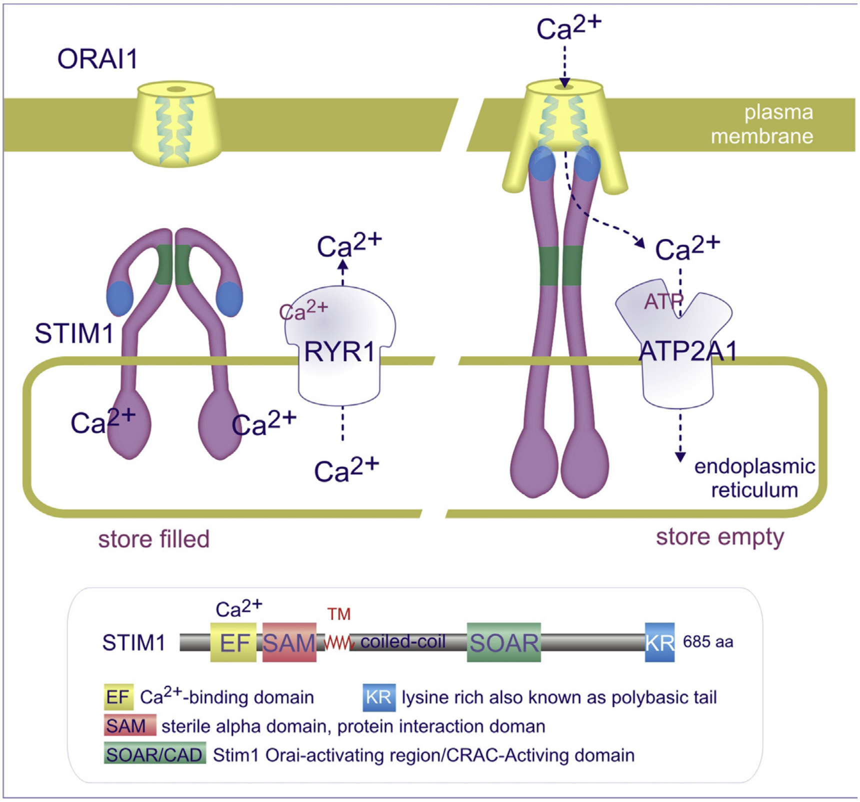


FIGURE 6-13 Proposed mechanism for STIM-mediated regulation of ORAI conductivity. The lack of Ca^{2+} in the store, as a consequence of repeated release through RYR/ITPR, brings about a switch-blade movement just above the SOAR region. This allows the lysine-rich motif (KR) to interact with transmembrane helices that constitute the pore of the ORAI1 channel and change its permeability. The Ca^{2+} pump (ATP2A1) replenishes the intracellular store.

ORAI and STIM came to light in studies with lymphocytes

We have elaborated the regulation of intracellular-free Ca^{2+} in skeletal and smooth muscle but the interest for ORAI and STIM1 originates in the understanding of immune-cell activation, in particular T lymphocytes (understanding clonal expansion and tolerance) (Gardner, 1989; Oh-Hora et al., 2008) and mast cells (understanding anaphylaxis) (Hoth and Penner, 1992; Baba et al., 2008). T lymphocytes require a sustained elevated level of Ca^{2+} in order to commit to a cycle of cell division in response to the recognition of an antigen, by the T-cell receptor, presented by a macrophage in the context of MHC. The elevated intracellular level of Ca^{2+} is initially entirely dependent on IP_3—mediated release from intracellular stores but a sustained signal (in the order of tens of minutes) requires an inward Ca^{2+} current, referred to as I_{CRAC} and that is where ORAI comes into play. We deal with the subject of T-cell activation in detail in Chapter 15, "Activating the Adaptive Immune System." The importance of Ca^{2+} entry through ORAI was emphasized by the finding that one of the hereditary forms of severe-combined immune deficiency (SCID), characterized by a lack of function of both B- and T-lymphocytes (Buckley, 2003), is the consequence of a loss-of-function mutation in ORAI (Feske et al., 2006; Vig et al., 2006). The protein was initially named CRACM, for Ca^{2+}-release activated Ca^{2+}-current channel modulator, as it was not yet clear whether the protein involved in the Ca^{2+} current (I_{CRAC}) modulated other Ca^{2+} channels, in particular the transient potential Ca^{2+} channels (TRPCs), or constituted a channel itself. The name ORAI, pronounced as "O reh," was coined in 2006 and refers to the keepers of the gates of heaven as described in the Iliad–book 5, by Homer (Greek mythology, not to be confused with Homer Simpson). Nearly at the same time as the molecular identity of ORAI came to light, it was proven unambiguously that STIM regulates its conductivity (Soboloff et al., 2006; Peinelt et al., 2006).

STIM stands for stromal-interacting molecule, because it was originally identified as a cell surface protein expressed on bone marrow stromal cells and playing a role in the regulation of proliferation of pre-B lymphocytes (Oritani and Kincade, 1996). In parallel, it was recognized as a protein of which lack of expression is associated with a number of pediatric malignancies, including Wilms tumor, rhabdoid tumors, and rhabdomyosarcomas (Parker et al., 1996; Sabbioni et al., 1997). In other words, STIM qualifies as a tumor suppressor. The role of STIM at the cell surface is not clear to us. Although discovered in non-excitable cells, by 2008 it was clear that both STIM1 and ORAI had their place in skeletal muscle and both play a universal role in excitable and non-excitable cells (Stiber et al., 2008).

Mast cells, first described by Paul Ehrlich in his doctoral thesis in 1878, are basophil-like granulocytes which make up part of the immune system. They often surround blood vessels and nerves and are prominent in skin, mucosa of the lungs, the digestive tract, and the mucosa and conjunctiva of the oral and nasal cavities. In response to an antigen, they secrete inflammatory mediators, such as histamine, thromboxane, prostaglandin D2, platelet-activating factor, and serine proteases. The antigen crosslinks type-E immunoglobulin (IgE) bound to FcεRI receptors which are expressed on the mast cell surface. If the antigen is also an allergen, the response can present a particular problem for allergic individuals such as sneezing, tearing, excessive mucus production, and sometimes a severe drop in blood pressure, anaphylaxis, because histamine is a potent vasodilator.

Rhabdomyosarcomes: rare form of cancer often occurring in childhood and arising from skeletal muscle progenitors that develops electively in areas where there are no skeletal muscles such as brain cavities and the genitourinary tract. "Sarco" (origin Greek) and "myo" (origin Latin) refer to the same anatomical structure: muscle. The significance of "rhabdo" is unknown to us.

Transient Receptor Potentials (TRPs) are ion channels that came to light in studies dealing with signal processing in the *Drosophila* compound eye (return to Chapter 5, "Sensory Signal Processing," Figure 5-12) (Montell, 2005). The human equivalents have roughly 40% sequence similarity and the channels concerned in Ca^{2+} movements in response to the production of IP_3 are called canonical TRP or TRPC. *Drosophila* also contributed to the further identification of ORAI, but this time with the use of S2 cells. Insect S2 cells exhibit a store-operated current that shares biophysical properties with the CRAC channels in human T lymphocytes. In a genome-wide screen using RNAi to knock down gene expression, candidates were identified that regulate the CRAC channel conductivity. Among 11 predicted transmembrane proteins, STIM and Olf186-F stood out as being essential for Ca^{2+} currents provoked by depletion of intracellular stores (blocking the endoplasmic reticulum Ca^{2+} pump with thapsigargin). Alignment of Olf186-F with mouse and human sequences revealed its similarity with the three ORAI genes. The study supported growing evidence that Olf186-F constitutes part of the CRAC channel and that Stim serves as the messenger for its activation (Zhang et al., 2006).

Inositol trisphosphate receptors

In 1983, in the face of a commonly held belief that mitochondria were the major intracellular Ca^{2+} stores, IP_3 was shown to release Ca^{2+} from non-mitochondrial stores in permeabilized pancreatic acinar cells (Streb et al., 1983). This finding identified the missing link in the sequence of events separating receptors in the plasma membrane from intracellular Ca^{2+} events. Subsequent studies showed that the IP_3 receptors are Ca^{2+}-permeant cation channels that open when they bind both IP_3 and Ca^{2+}. The human genome contains three genes, ITPR1–3, giving rise to three different proteins, with a molecular mass of ~310 kDa each and which assemble into homo- or hetero-tetrameric structures to form functional channels that have the appearance of a mushroom (Figure 6-14) (Sato et al., 2004) They rank, together with RYR, among the largest ion-channel complexes. They are expressed in nearly all mammalian cells and occur mainly in the membrane of endoplasmic reticulum but are also detected in Golgi

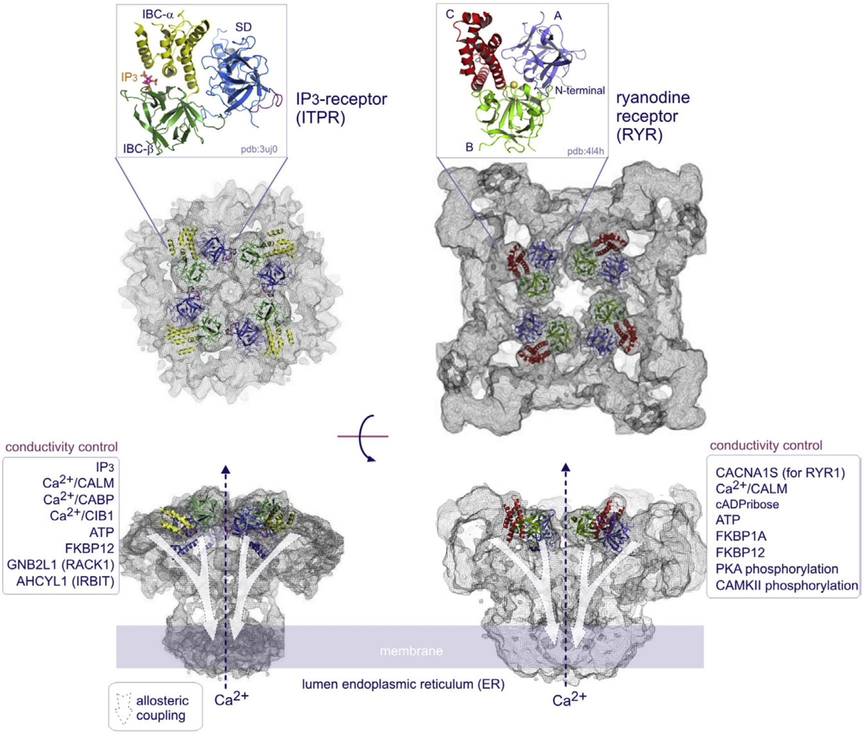


FIGURE 6-14 Three-dimensional reconstruction of the whole assembly of IP_3 and ryanodine receptors obtained by cryo-electron microscopy. Images of ITPR are adapted from Seo et al. (2010) and images from RYR are adapted from Tung et al. and kindly provided by Dr Filip van Petegem, University of British Columbia, Canada (Tung et al., 2010).

vesicles (return to Figure 6-1). Electrophysiological studies of recombinant ITPRs expressed in native membranes indicate that all three gene products have very similar gating and ion-permeation characteristics (Mak et al., 2000) but may vary with respect to Ca^{2+} regulation, tissue expression, and subcellular localization (Dupont et al., 2007).

IP_3 receptors are spatially organized into clusters of up to about 15 channels, which are scattered across the ER membrane with distances of 1–7 μm. The IP_3 receptor functions in a Ca^{2+}-induced Ca^{2+}-release mode (sometimes referred to as CICR). This means that cytosolic Ca^{2+} must be present at a minimum concentration of 100–200 nM in order to allow Ca^{2+} to pass the channel pore and the subsequent increase in Ca^{2+} will boost the release even further. Given that the diffusion length of cytosolic free Ca^{2+} is generally less than 2 μm (slow diffusion because of numerous Ca^{2+}-binding proteins and rapidly removed by pumps or exchangers), the activation coupling between channels within a cluster is much stronger than the coupling between channels in adjacent clusters (Thurley et al., 2012). The particularity of the channel is that when the local level of Ca^{2+} rises above a certain concentration it next closes the channel. This is explained, hypothetically, by the presence of two Ca^{2+} binding sites on the calmodulin that interacts with a suppressor domain (SD): one with high affinity, allowing the passage of Ca^{2+} through removal of the suppressor; the other with low affinity, blocking the passage. As local Ca^{2+} levels drop, the channel reopens and a cycle of closed and open state ensues (Hajnoczky and Thomas, 1997). Importantly, the binding of IP_3 shifts the inactivating concentration of Ca^{2+} to a higher level and as a consequence the channel remains open for a longer time (Mak et al., 1998; Dupont et al., 2007). Openings of single IP_3 receptors give rise to "blips," they may trigger collective openings of IP_3 receptors within a cluster, causing "puffs," while Ca^{2+} diffusing from a puff-site can activate neighboring clusters and involving RYR channels, eventually leading to a global "spike" (Figure 6-15) (Thurley et al., 2012). It is important to note that plasma membrane ion channels will also contribute to the scale and duration of the Ca^{2+} wave. The initial positive feedback of Ca^{2+} on Ca^{2+} release (CICR) underlies the regenerative nature of many Ca^{2+} signals.

The relative position of three domains in the N-terminal segment, domains IBC-α, IBC-β (inositol binding core), and SD (suppressor domain) play a crucial role in all this (Taylor et al., 2004). Inositol-1,4,5-trisphosphate, which binds at the interface of the two IBC domains brings about a conformational change which, through allosteric coupling, reduces the inhibitory action of high Ca^{2+} concentrations and increases the conductivity of the channel pore (Figure 6-14) (Taylor et al., 2014). Given the immense size of the channel, it is not yet clear how the different channel segments are functionally (allosterically) connected.

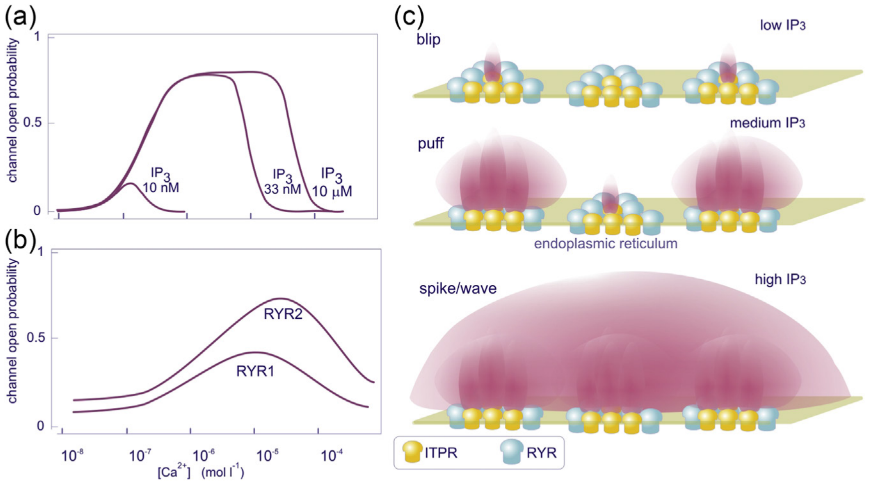


FIGURE 6-15 **Biphasic Ca^{2+}-induced Ca^{2+} release from ITPR and RYR channels.** (a) This graph shows the open probability of IP_3 receptors in the outer membrane of nuclei of *Xenopus laevis* oocytes at different levels of IP_3. Note that at low levels of IP_3, the release window is very narrow and this means that very little Ca^{2+} will flow out of the endoplasmic reticulum. The inhibitory Ca^{2+} concentration shifts to the right when IP_3 levels increase, allowing channels to remain open for a longer period of time. Graph adapted from Mak et al. (1998), (b) Ca^{2+}-induced Ca^{2+}-release characteristics of ryanodine receptors type-1 and -2. Graph adapted from Capes et al. (2011). (c) Ca^{2+} release comes in different patterns. Low IP_3 gives rise to blips, medium levels to puffs, and high levels to spikes or waves (occurring when clusters of IP_3 receptors stimulate adjacent clusters of IP_3 receptors as well as ryanodine receptors). Inward Ca^{2+} currents through plasma-membrane channels contribute to the generation of spikes and waves.

Besides the already mentioned regulation mechanism by Ca^{2+}/calmodulin, the gating of the channel can be modulated by numerous other factors such as binding of cAMP, ATP, CABP, CIBP, FKBP12, GNB2L1, and AHCYL1. In addition, there is evidence that the receptor has several distinct types of Ca^{2+}-binding sites, including at the luminal side, which means that the Ca^{2+} concentration in the endoplasmic reticulum may also have a say in the regulation of channel conductivity (Taylor et al., 2014).

The ryanodine receptor

Ryanondine receptors are even larger; they are assemblies of four 560 kDa subunits arranged in a homotetrameric structure also giving rise to a mushroom-shaped channel (Figure 6-14) (Ludtke et al., 2005). Three genes exist, RYR1–3, of which RYR1 is predominant in skeletal muscle but also expressed in brain. In contrast, RYR2 is abundantly expressed not only in cardiac muscle (Tunwell et al., 1996) but also detected in smooth muscle, as well as in the brain and placenta, whereas RYR3, although initially discovered in the brain (Nakashima et al., 1997), is expressed in the brain, skeletal muscle, liver, and kidney.

Ryanodine is a plant alkaloid extracted from *Ryania speciosa* and further developed as an insecticide (ryanoid formulations were patented as Ryanex). It was found to bind, with very high affinity, to Ca^{2+} channels in all types of muscle and was subsequently used for its purification (hence the name Ryanodine receptor) (Imagawa et al., 1987). At nanomolar concentrations it locks the Ca^{2+} channel in half-open state, causing muscle spasm, whereas it fully closes them at micromolar concentration, causing paralysis.

Ryanodine receptors exhibit the same Ca^{2+}-induced Ca^{2+}-release characteristics as described above (Figure 6-15) with the exception that they lack an IP_3-binding site. Like the ITPR, gating of the channel is controlled by binding of ATP and cADPribose, by phosphorylation by PKA and CAMKII and by a number of proteins that attach to the receptors such as FKBP1A and FKBP12 (Figure 6-14). As we have already shown, in the skeletal muscle, RYR1 physically interacts with and its permeability is controlled by the dihydropyridine-sensitive CACNA1 channel (also known as DHPR or voltage-sensitive L-type Ca^{2+} channel). It is still unclear how the proteins modulate each other's Ca^{2+} permeability (Rebbeck et al., 2014). It is important to remember that unlike cardiac-type excitation–contraction coupling, skeletal-type excitation–contraction coupling does not require such Ca^{2+} influx for the opening of RYR1.

The N-terminal domain of the RYR and ITPR channels are structurally and functionally conserved. The arrangements of the A, B, and C domains of RYR resemble those of the IBCα, IBCβ, and SD domain of ITPR (compare the inserts in Figure 6-14). Moreover, the A-domain of RYR can functionally replace the SD domain in ITPR and more strikingly, grafting the N-terminal domains of ITPR onto the C-terminal segment of RYR gave rise to an IP_3- and ryanodine-sensitive chimer (Seo et al., 2010). Like for the ITPR it is proposed that the N-terminal domains acts as a "brake" and it is also proposed that inter-domain contacts between the A, B, and C domain play an important role in regulation of channel opening. A number of mutations occur in these domains and they lead to inappropriate functioning of the channel as manifested by ventricular tachycardia (causing cardiac arrhythmias) and idiopathic ventricular fibrillation (for RYR2) and malignant hyperthermia and central core disease (for RYR1). These mutations affect the inter-domain interface as well as the binding of a chloride ion (Kimlicka et al., 2013a,b).

Cryo-electron tomography (CET) is a type of electron microscopy where images are taken of frozen sections tilted at numerous different angles. Compiling the numerous 2D images allows for a 3D reconstruction of the object. Typically resolution is in the range of 50 to 15 Å, depending on the quality of the images and the processing of the tomograms. In the case of the ryanodine and IP_3 receptors, the X-ray crystallographic data (resolution at ~3 Å) of the N-terminal segment are projected on the tomographic representation of the channel. CET is a valuable tool in the reconstitution of big molecular complexes such as the RYR and ITPR channels, nuclear pores, ribosomes, or proteasomes.

Spatiotemporal regulation, Ca^{2+} microdomains, and global cellular signals

The interplay between the above described Ca^{2+}-regulating agents gives rise to a variety of optical phenomena that may be observed when we study cells with fluorescent Ca^{2+} indicators. These range from "Ca^{2+} sparks," the punctate flashes seen in cardiac muscle cells (lasting only tens of milliseconds), to broad waves that spread within seconds right across the cytoplasm. Ca^{2+} waves have been observed in a wide range of non-excitable cells that include pancreatic acinar cells, smooth muscle cells, megakaryocytes, astrocytes, or fertilized *Xenopus* oocytes. Digital imaging techniques and confocal microscopy have added considerable detail to our knowledge of Ca^{2+} signals. Within cells, the initial increments in Ca^{2+} concentration tend to be confined to subcellular micro-domains. These tend to lie close to locations where effector molecules are gathered and where action takes place, for example in the vicinity of the triad structures in skeletal muscle or in neurons near to neurotransmitter release sites or confined within individual dendritic spines. These localized increases in Ca^{2+} may remain restricted and transient or they may be amplified by Ca^{2+}-induced Ca^{2+} release and then spread further. They may eventually merge to generate a global increase in Ca^{2+} concentration which permeates the whole cell, especially under conditions of strong stimulation, and may last for minutes or longer. Apart from the intimate interplay between the endoplasmic reticulum and ion channels in the plasma membrane, the close proximity of mitochondria, lysosomes, and Golgi vesicles also plays a role. Their transporters can remove Ca^{2+} and rapidly silence the signal, and therefore influence the propagation of not only the signal but also the frequency of the oscillations. They may do the reverse and contribute to the propagation of the signal or to a sustained elevated level of Ca^{2+} by feeding the cytoplasm with Ca^{2+} (Duchen, 2000; Boitier et al., 1999; Takeuchi et al., 2013) (Figure 6-11).

Oscillations occur in systems where feed-forward or feedback control occurs with a certain delay. Essential factors in the generation of Ca^{2+}

oscillations are the biphasic gating control of the IP_3 and ryanodine receptors, the removal of Ca^{2+} by pumps, and the cyclic presence of IP_3, each of which are under the control of Ca^{2+}. All phospholipase C isoforms require Ca^{2+} for their catalytic action and elevated levels of Ca^{2+} may thus augment the number of competent enzymes (return to chapter 3, Figures 3-16 and 3-18). The isoforms PLCδ and -ζ require Ca^{2+} for membrane binding through their C2 domain and they have a functional EF domain (Rhee, 2001). The three isoforms of the enzyme that removes IP_3, inositol-1,4,5-trisphosphate kinase (ITPKA, -B, -C), are activated by Ca^{2+}/calmodulin and the Ca^{2+} pumps are controlled by Ca^{2+}. Moreover, the oscillations of the IP_3 and RYR channels are modulated by allosteric factors described above. Which of these factors determine the frequency of the oscillations is not easy to dissect and they may vary between cell types. For instance, studies with cells loaded with photo-releasable IP_3 have revealed that in pancreatic acinar cells, acetylcholine-induced Ca^{2+} oscillations depend on IP_3 oscillations, whereas Ca^{2+} oscillations induced by the same stimulus in airway smooth muscle occurs independent of IP_3 and are the consequence of the biphasic Ca^{2+} control of IP_3 receptors (Sneyd et al., 2006).

Intercellular Ca^{2+} waves

Calcium waves propagate not only into the cell but also between cells. In non-excitable cells the process occurs through diffusion of IP_3 into neighboring cells through Gap junctions. As an alternative, propagation occurs in a paracrine mode, where the target cells produce a mediator that diffuses into the tissue and provokes receptor-mediated Ca^{2+} signals. Naturally, both mechanisms may act in concert and generate a strong and widespread wave (Figure 6-16) (Dupont et al., 2007). In excitable cells the tissue waves are caused by the propagation of an action potential, such propagation occurs in the heart and in smooth muscle cells of the gut (peristaltic wave).

DECODING Ca^{2+} OSCILLATIONS

In most non-excitable tissues the Ca^{2+} signal oscillates and the frequency, rather than the amplitude, correlates with the signal strength. This has been observed in smooth muscle contraction (Perez and Sanderson, 2005) (return to Chapter 4, Figure 4-19) and in gene expression in B-lymphocytes (Dolmetsch et al., 1998). In pancreatic acinar cells, low frequency Ca^{2+} oscillations, as induced by intermediate levels of acetylcholine, are a more potent stimulus for enzyme secretion than high frequency oscillations or an elevated steady-state level (Kasai and Augustine, 1990). The question remains largely unanswered of how effector proteins deal with oscillations. A possible mechanism is given by a study with Ca^{2+}/calmodulin-dependent protein kinase 2A (CAMK2A). The enzyme is composed

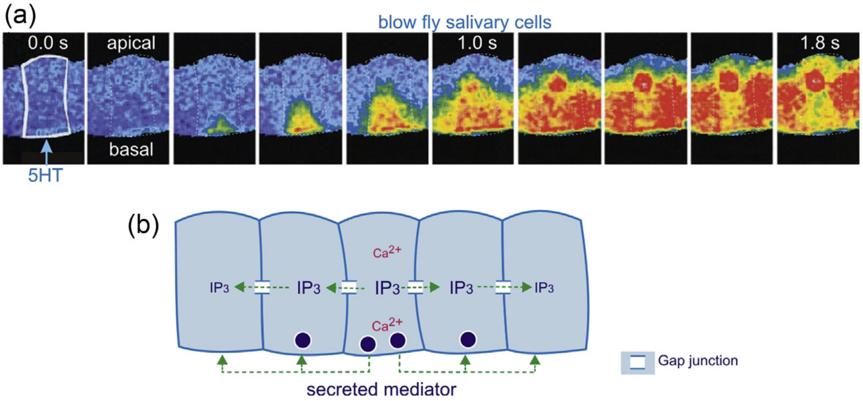


FIGURE 6-16 **Calcium waves that propagate into the surrounding cells.** (a) Confocal images of blow-fly salivary cells exposed to a low dose (3 nM) of 5HT applied to the basal surface of the cells (in a Ca^{2+}-free medium). The central cell is outlined in white. The transient lasts for about 10 s and progresses from the basal membrane to the apical surface and into the neighboring cells. *Image obtained with permission from Zimmerman (2000).* (b) Propagation of Ca^{2+} waves can occur through diffusion of IP_3 via Gap junctions or through Ca^{2+}-mediated secretion of a mediator that subsequently provokes receptor-mediated Ca^{2+} signals. Both mechanisms may act in concert. The diffusion of Ca^{2+} across cells is generally considered too slow, because of the abundance of buffering Ca^{2+}-binding proteins, to cause intercellular waves.

of a catalytic, regulatory, and hub domain, the latter two being connected by a hinge region. It occurs in the cell as a dodecameric assembly (holoenzyme) (Hoelz et al., 2003) in which the individual protein kinases oscillate between a compact (hub and kinase domain close) and extended (hub and kinase separated) auto-inhibited state (Figures 6-17 and 6-18). Ca^{2+}/calmodulin cannot bind the compact state but has access to the extended state. By binding the regulatory domain/hinge region, it prevents not only the return to the compact state but also removes an auto-inhibitory constraint, allowing the kinase to phosphorylate Thr286 on its neighbor and this constitutes the activation mechanism. In order to effectively activate the kinase complex numerous catalytic domains must simultaneously pop out of the compact state during the brief presence of Ca^{2+} (otherwise phosphorylation *in trans* cannot proceed). Once phosphorylated on Thr286, CAMK2A retains its activity and no longer requires the presence of Ca^{2+}/calmodulin. Only a threonine/serine phosphatase can return the enzyme to its inactive state. Phosphorylated CAMK2A plays an important role in the acquisition of long-term potentiation (LTP) (Malenka and Bear, 2004) as it was shown that transgenic mice which express a kinase mutant that cannot be phosphorylated on thr286 display impaired spatial learning (Silva et al., 1992a,b).

There are four CaMKII genes (denoted CAMKA, -B, -D, and -G) in humans. The enzymes produced by these genes have virtually identical kinase

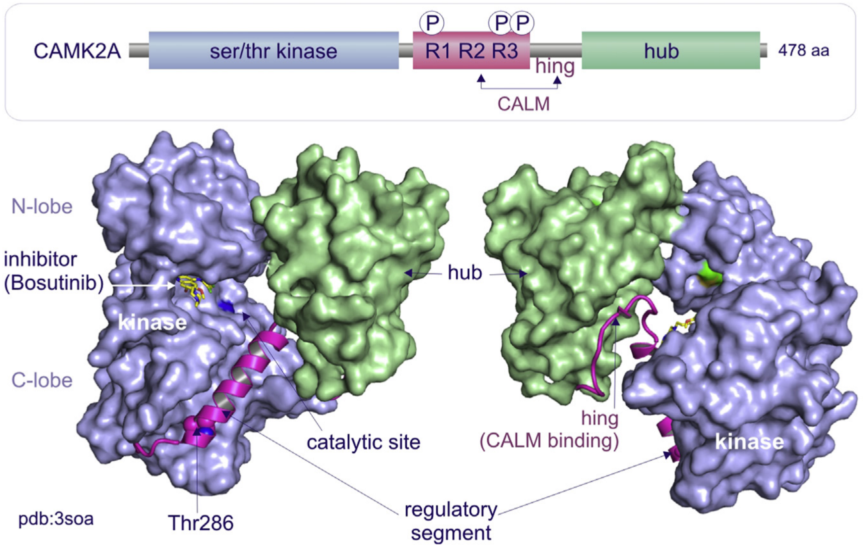


FIGURE 6-17 Domain architecture and molecular structure of CAMK2A. CAMK2A has been crystallized with the kinase inhibitor Bosutinib (shown in yellow). Note the three segments: the kinase domain (light blue), the regulatory helix and hinge (pink), and the hub (green). Ca^{2+}/calmodulin binds at the interface off the regulatory segment (helix) and the hinge region.

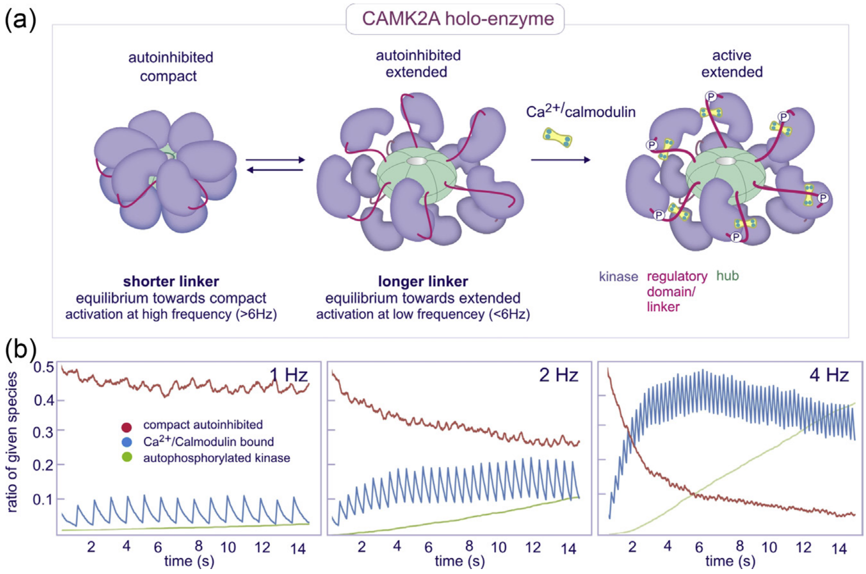


FIGURE 6-18 **Composition of the holo-enzyme and its regulation by Ca^{2+} oscillations.** (a) Schematic representation of the holo-enzyme (octadecamer) in a compact or extended auto-inhibited form and in an extended active form after binding of Ca^{2+}/calmodulin and phosphorylation of Thr286. (b) Computer simulation of the relationship between the Ca^{2+} oscillation frequency and activation of a long linker CAMK2 as monitored by the phosphorylation of Thr286. Note that for the given protein-kinase characteristics, different Ca^{2+} oscillation frequencies have different impacts on activation. At 1 Hz, repeated opening of the kinase occurs but does not lead to long-term activation, whereas activation is efficient at 4 Hz. Figures adapted with permission from Chao et al. (2011).

domains (95% sequence identity) and similar hub domains (80% identity). The principal difference between these gene products is in the linker connecting the kinase domain to the hub domain, which is variable in both sequence and length. At least 38 distinct mammalian splice variants are generated by lengthening or shortening this region (Tombes et al., 2003). The length of the hinge region influences the state of the kinase complex; short linker kinases more often occur in a compact state whereas long linkers more often occur in an extended state (Figure 6-18) (Bayer et al., 2002; Chao et al., 2011). It was shown in a computer simulation that the compact state is most effectively activated by high-frequency Ca^{2+}oscillations whereas the extended state reaches maximum activity at lower Ca^{2+} oscillations. Changing the frequency of Ca^{2+} spikes alters the spectrum of activated CAMK2 isoforms and this, in part, may explain why signal strength is coded in spike frequency and not in the amplitude of the Ca^{2+} signal. If different isoforms occur at different subcellular localizations, the possibility arises where frequencies not only determine the level of activation of the protein kinase but also the type of response (as a consequence of access to different substrates). The universality of this regulation mechanism still needs to be clarified.

MOBILIZING Ca^{2+} THROUGH CYCLIC ADP RIBOSE, NAADP, AND SPHINGOSINE-1-PHOSPHATE

While IP_3 is the most widespread and certainly the best understood Ca^{2+}-mobilizing second messenger, there are other small molecules that can also cause the release of Ca^{2+} from intracellular stores. Two of these are adenine nucleotides, cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP), and the third sphingosine-1-phosphate. The adenine nucleotides were first identified as Ca^{2+}-mobilizing agents in sea urchin (Galione et al., 1991; Lee and Aarhus, 1995) and (in mammalian cells) both are the product of the membrane enzyme CD38, using the substrates NAD^+ and $NADP^+$, respectively (Lee, 2006). Spingosine-1-phosphate was first discovered in permeabilized hamster smooth muscle cells (Ghosh et al., 1994). The structures are shown in Figure 6-19. In contrast to IP_3, it has been difficult to establish their credentials as bona fide messenger molecules in mammalian cells in particular because their intracellular receptors remain elusive.

Cyclic ADP-ribose

Evidence for Ca^{2+} mobilization by cADPR exists for a range of cell types that includes oocytes, neuronal cells, muscle cells (skeletal, smooth, and cardiac), certain secretory cells, and lymphocytes (Guse, 2004). When cADPR is introduced into cells at low concentrations (10–50 nM), it initiates release of Ca^{2+} from a ryanodine-sensitive pool. However, it is unlikely

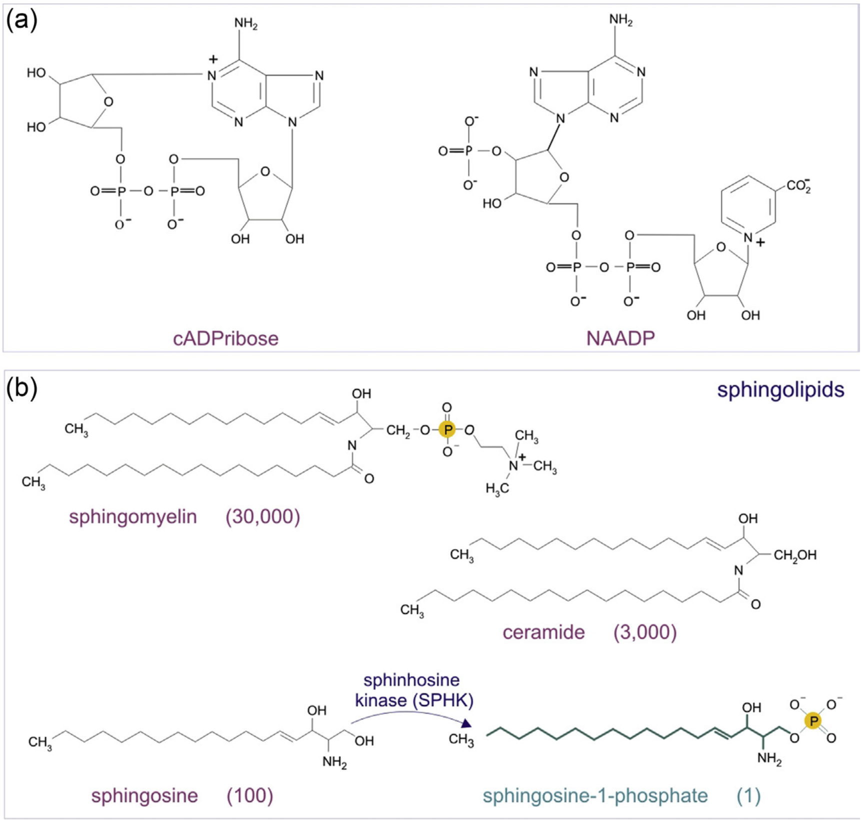


FIGURE 6-19 **Structures of cyclic ADP-ribose, NAADP, and sphingolipids.** (a) cADPribose liberates Ca^{2+} from the endoplasmic reticulum in a fashion that requires RYR. NAADP liberates Ca^{2+} by gating the two-pore channels TPC1–3. (b) Different sphingolipids detected in cell membranes. Sphingosine-1-phosphate can escape from the membrane and acts either as an extracellular ligand that binds to a specific receptor, S1 PR, or as an intracellular ligand that binds an as yet unidentified receptor. In both cases it causes the liberation of Ca^{2+}, as an extracellular ligand through G-protein-mediated activation of phospholipase C, and as an intracellular ligand through direct liberation from the endoplasmic reticulum. The figures behind the different sphingolipids indicate their relative abundance.

that it directly binds the RYR and almost certainly requires an accessory protein (Venturi et al., 2012).

Activation of ADP-ribosyl cyclase to form cADPR occurs in response to a cell surface ligand–receptor interaction, but how these events are coupled is unclear and there is uncertainty about the location of the enzyme. The only well-characterized form of ADP-ribosyl cyclase is CD38, an enzyme present on the extracellular surface of the plasma membrane. Curiously, it was shown to occur in two opposing orientations, one with the catalytic center at the outside and one at the inside of the cells (Zhao et al., 2012).

The clearest evidence for the formation of cADPR and for its action as a second messenger comes from pancreatic β cells. In response to a glucose stimulus, the level of intracellular cADPR rises and this is accompanied by elevation of cytosol Ca^{2+} followed by insulin secretion (Kato et al., 1999). Also, stimulation of T lymphocytes through their T-cell receptor complexes (refer chapter "non-receptor tyrosine kinases and lymphocyte activation") activates ADP-ribosyl cyclase to produce a sustained elevation of the level of cADPR and a long-lasting Ca^{2+} signal (a series of waves) (Guse et al., 1999). Sustained Ca^{2+} elevations are necessary for lymphocytes to become committed to a full proliferative response.

Nicotinic acid adenine dinucleotide phosphate

Although NAADP was discovered later than cADPR was discovered, there is now better evidence linking NAADP to a range of physical process in mammalian tissues. It has been shown that its level in acinar cells from the pancreas (exocrine secretory cells) is in response to the peptide hormone cholecystokinin (Yamasaki et al., 2005). Further direct evidence for a second messenger function for NAADP has been obtained from studies of T lymphocytes. When these cells are activated through the T-cell receptor/CD3 complex, there is an approximate sevenfold increase in the level of NAADP lasting some 20 s, followed by a slower component that lasts for several minutes. The transient increase is accompanied by an increase in cytosolic $[Ca^{2+}]$ that peaks after a few minutes. The mechanism by which NAADP is synthesized is still poorly understood but it liberates Ca^{2+} from early and late endosomes by opening of two-pore channels (TPC1–3) (return to Figures 6-1 and 6-12) (Calcraft et al., 2009). The liberation of Ca^{2+} in turn activates a Ca^{2+}-induced Ca^{2+} release through the RYR (or ITPR) when the TPC-containing organelles are near the endoplasmic reticulum.

Elevation of Ca^{2+} by sphingosine-1-phosphate

Many lipids have key roles in regulatory mechanisms either as substrates of receptor-activated effectors, as tethering points for signaling proteins or as membrane-resident second messengers. They include for instance $PI(4,5)P_2$, $PI(3,4,5)P_3$, diacylglycerol, arachidonic acid, and lysolipids. Classification of this group as "biologically active" has meant that changes in their levels or locations affect cell function. To this list must be added the sphingolipids—the precursors and metabolites of the membrane lipid sphingomyelin. Because these compounds through their metabolism are readily interconvertible, and because they are mostly membrane restricted, they give rise to a highly complex network of regulatory pathways that feature prominently in huge-range cellular processes, many of which are pathophysiological (Hannun and Obeid, 2008).

The structures of the most important sphingolipids are shown in Figure 6-19. Ceramide lies at the center of the network and through variations in saturation, chain length, and substitution, exists in some 50 distinct molecular forms. It participates in numerous pathways; most notably in cellular stress responses. Sphingosine, the single chain lipid formed from ceramide is less hydrophobic and so can move between different cell membranes, where it affects multiple protein targets (e.g., PKC). Phosphorylation of sphingosine produces the amphipathic sphingosine-1-phosphate (S1P), which is even more soluble. It exists at low (nanomolar) levels in resting cells and it can transfer across the plasma membrane through ABC-C1 transporters. Outside cells it binds to high affinity G protein-coupled receptors (S1RPR1–5) and has roles in cell growth, survival and migration, and it is important in inflammatory processes. Inside cells it acts as a Ca^{2+} mobilizing messenger. The intracellular levels of sphingo-1-phosphate are tightly regulated. They can never rise too high, in part because its surface active properties would disrupt membranes. Because its concentration is kept low, it must act on Ca^{2+} stores through high affinity receptors though these remain to be identified (Ghosh et al., 1994; Meyer-zu-Heringdorf et al., 2003; Spiegel and Milstien, 2003).

ABC transporters are ATP-binding cassette transporter proteins that use ATP hydrolysis to carry impermeant molecules across membranes. Examples are the multidrug resistance transporters (MDRs) which eliminate numerous toxic substances from cells including a wide range of chemotherapeutic agents and thus confer drug resistance.

Ca^{2+} IN ACTION

Exocytosis of neurotransmitters

When studies on the molecular mechanisms of exocytosis started in earnest, around 1980, the sequence of events that constitute neurotransmitter release was described as the process where membrane depolarization opens specific “calcium gates” in the terminal axon membrane which leads to an influx of calcium ions. Having reached the internal surface of the membrane these calcium ions, next, initiate the quantal release reaction. As Bernard Katz describes in his Nobel Lecture, how exactly calcium ions bring about neurotransmitter release, within a time span of only few microseconds, was not known at that time (and it still seems an issue today):

> Up to this point of the argument [action potential followed by the entry of Ca^{2+}] we are on reasonable firm ground established by electrophysiological experiments. Beyond this point, I must draw on converging observations from ultrastructural and biochemical studies, all of which go to make up a plausible and, I think, very strong hypothesis, namely that the quanta of transmitter molecules are enclosed within

> synaptic vesicles which undergo frequent transient collisions with the axon membrane, that calcium brings about attachment and local fusion between vesicular and axon membranes, and that this is followed by all-or-non discharge of the vesicular content into the synaptic cleft. ***Katz (1970) and Katz and Miledi (1969a,b)*).**

The challenges for the secretory mechanism are twofold: first, how to get two membranes so close that they fuse and, second, how to control the timing of the event. The proteins that bring together two membranes are described as the SNARE complex. The essential components of this complex are two transmembrane proteins, one located on the synaptic vesicle, synaptobrevin, and one on the plasma membrane, syntaxin, and one lipid-anchored protein, named SNAP25 and also located at the plasma membrane. Syntaxin is complexed with Munc18. Progressive supercoiling of the coil regions of the SNARE proteins reduces the distance between the vesicle and plasma membrane, until the two fuse and create a fusion pore through which the neurotransmitter is released (Figure 6-20) (Sudhof and Rothman, 2009). Secretion vesicles are recruited to the plasma membrane, at the so-called active zone of the pre-synaptic membrane, by RIM and Rab3/Rab27, and clamped into a primed complex through a partly assembled SNARE complex, stabilized by Complexin. Importantly, both syntaxin and RIM (Kaeser & et al., 2011) play a role in the recruitment of the voltage-gated Ca^{2+} channels into the active zone (members of the CACNA1 family). The Ca^{2+} sensor of the system is synaptotagmin, a transmembrane protein located on the vesicle. It contains two Ca^{2+}-binding domains—C2A and C2B (Perin et al., 1990; Sudhof, 2013). Deletion of the C2B domain greatly disrupts neurotransmitter release, whereas the loss of C2A, or the loss of its Ca^{2+}-coordinating aspartates, has hardly any effect (Stevens and Sullivan, 2003; Chapman, 2008). How Ca^{2+} provokes membrane fusion is still not certain. One of the unresolved enigmas is the observation that neurotransmitter release precedes the influx of Ca^{2+} into the synaptic terminal.

Two complementary mechanisms are proposed to account for the requirement of Ca^{2+}. The first concerns its role in the priming of the SNARE complex and the second in the induction of the membrane fusion. The first requires the entry of Ca^{2+} through the voltage-gated Ca^{2+} channel, whereas the second only requires Ca^{2+} binding in the channel pore (Atlas, 2013). With respect to priming of the SNARE complex, a recent study has shown that the C2B domain of synaptotagmin binds phosphatidyl-4,5-bisphosphate (PIP_2) in the plasma membrane, where it is abundantly present because of its sequestration by syntaxin. Binding to PIP_2 increases the Ca^{2+} affinity of the C2B domain by more than 40-fold. The entry of Ca^{2+} next creates a second-membrane-binding site, this time through Ca^{2+}-mediated attachment of C2B to acidic phospholipids (predominantly phosphatidylserine) in the neurotransmitter vesicle (Hinigmann et al.,

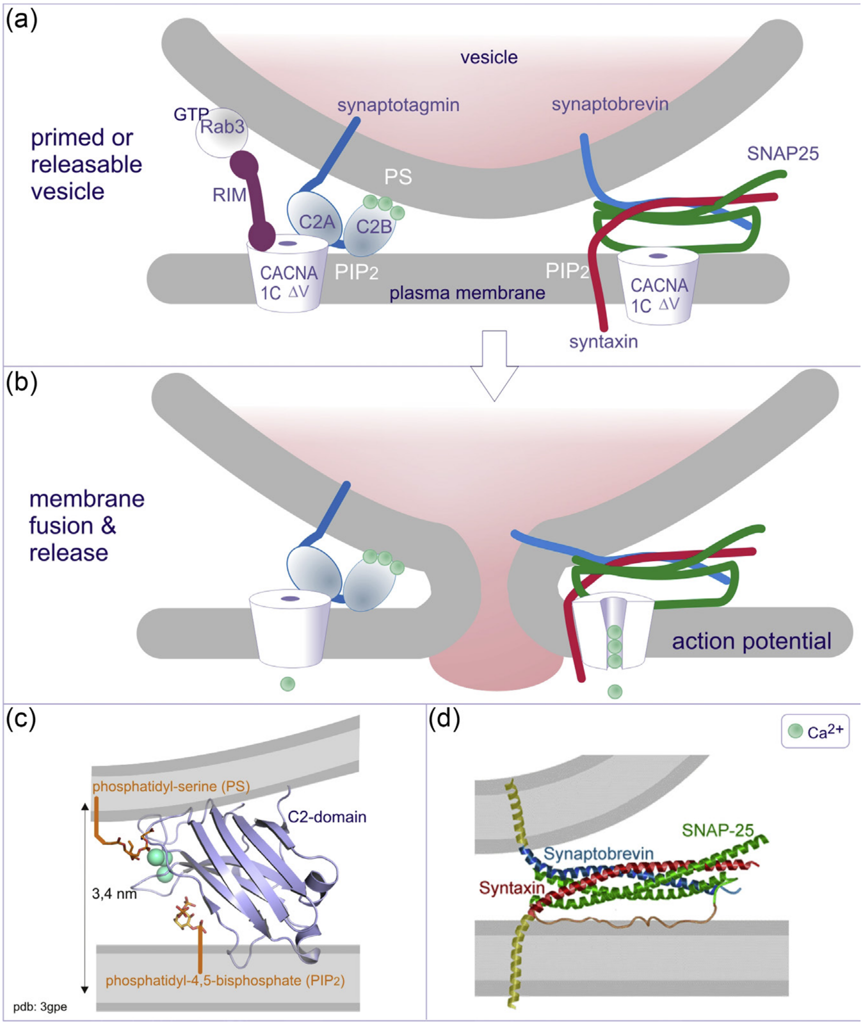


FIGURE 6-20 **Ca^{2+} and its role in neurotransmitter release.** (a) Entry of Ca^{2+} is required to prime the fusion complex. In this condition, the two membranes are very close, held together by the coiled-SNARE complex and by C2B. In its Ca^{2+}-bound state it interacts with phosphatidyl serine (PS) of the vesicle and with phosphatidylinositol-4,5-bisphosphate (PIP2) of the plasma membrane. Both RIM and syntaxin play a role in the recruitment of the voltage-sensitive Ca^{2+} channel (CACNA1C). (b) Pore fusion is thought to occur as a consequence of binding of three Ca^{2+} ions inside the selectivity filter of CACNA1C which brings about a change in the interaction with synataxin. How this causes membrane fusion is not clear. (c) Details of how the C2 b domain binds both PS and PIP2. Pdb 3gpe (with the addition of PS). (d) Coiled-coil structure of the SNARE complex (synaptobrevin, syntaxin, and SNAP-25).

2013). The distance between the two membranes is now reduced to a few nm (as observed by cryo-EM). The exocytosis complex is in a primed or releasable state. With respect to the fusion signal, membrane depolarization causes binding of three Ca^{2+} ions in the selectivity filter of the voltage-sensitive human Cav2.1 channel (CACNA1A). The binding is transmitted to the exocytotic machinery through interaction of the channel with the transmembrane region of syntaxin-1A (STX1A). How this causes fusion is not known.

The proposed model suggests that upon arrival of an action potential at the synapse, the Ca^{2+}-primed (releasable) vesicle fuses with the membrane within 100–1000 μs (microseconds). Fusion is triggered in response to a conformational change initiated at the pore of the Ca^{2+} channel and propagated to the exocytotic machinery via syntaxin. Concomitantly, Ca^{2+} that permeated through the channel into the cell during channel stimulation binds as yet unoccupied synaptotagmin-1, thus priming new secretion vesicles (an event that takes milliseconds rather than the microseconds observed for secretion). These primed vesicles only release their contact at the next action potential (Bachnoff et al., 2013).

James Rothman, Randy Schekman, and Thomas Südhof were awarded the Nobel Prize in Physiology or Medicine of 2013 “for their discoveries of machinery regulating vesicle traffic, a major transport system in our cells.” http://www.nobelprize.org/

Directed cell migration and spatiotemporal control

Migration is a fundamental property that enables organisms to develop (cells detach from each other, migrate elsewhere and form new embryonic layers), repair their tissues (progenitor cells migrate into existing tissues), and defend themselves against pathogens (neutrophils chase and engulf bacteria or lymphocytes home into lymph nodes). Cell migration also lies at the heart of metastasis of cancer cells. The migration process we illustrate in this section is referred to as protrusion–retraction, first described in detail by Abercrombie in 1970 (Abercrombie et al., 1970). It is characterized by a series of observations such as flattening of the cells, increased polarity (asymmetric position of nucleus and asymmetric distribution of cell mass), and an increased protrusion area (the leading lamella). In the process of migration, the cell makes new adhesion contacts with the substratum at the leading edge and detaches and retracts at the rear.

The term retraction is used for two different observations. First, retraction of the rear end of the cells and second, retraction of protrusions. In a Petri-dish environment, cells constantly not only throw out protrusions but they also retract and as a result the cells do not move or move slowly when the retraction surface is inferior to the preceding protrusion. Even migrating cells fully retract their protrusions from time to time and this may be one mechanism by which the direction of movement is determined. As shown in Figure 6-21, the migrating cell has two predominant lamellae.

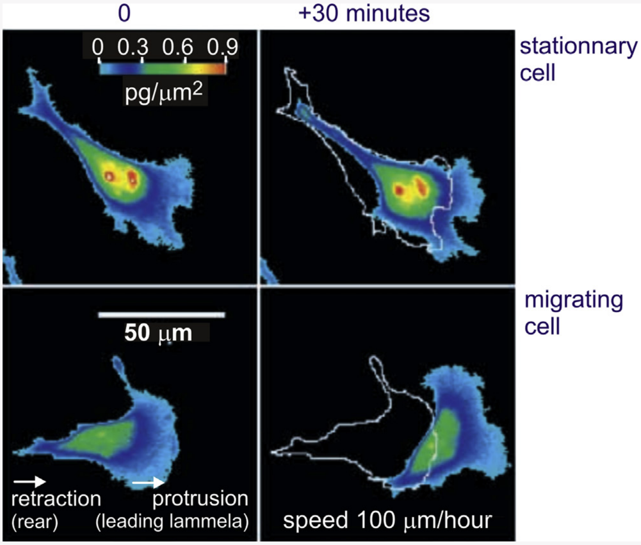


FIGURE 6-21 Shape changes that occur in migrating cells are flattening, asymmetric distribution of the nucleus and of cell mass, as well as the formation of large flat membrane protrusions (leading lamella). Cell thickness (pg/μm^2) is measured as the amount of protein encountered by the light source as it penetrates the cell. The cell treated with transforming growth factor-β1 (TGFβ1) has moved some 50 μm in half an hour (on average they move at 56 μm per hour) (Zicha et al., 1999).

Below we provide a “generic description,” a compilation of data that have been obtained from many different studies performed with different cell types, each having their own specificities. Migration involves the cytoskeleton of which the actin cytoskeleton has been studied in particular. We ignore the role of microtubules and intermediate filaments (keratin or vimentin). We highlight two phenomena that occur in the process of migration: contraction of arc-shaped dorsal actin stress-fibers in the lamella (composed of γ-actin) (Burnette et al., 2014) and the formation of actin-filament meshworks (composed of β-actin) at the leading edge (Pollard and Borisy, 2003; Karakozova et al., 2006).

The flattening of the lamella is a consequence of the shortening of the arc fibers (Burnette et al., 2014). These are attached to dorsal stress fibers, which in turn are fixed to focal adhesion sites (clusters of intergrin-type adhesion molecules attached to the extracellular matrix). Shortening the arc causes a downward movement of the dorsal fibers because this is the only way in which the distance between the arcs and the focal adhesions can be shortened (see Figure 6-22). Arc fibers are composed of antiparallel actin filaments, hold together at their extremities by α-actinin and interspersed by myosin dimers.

At the level of the leading edge, a new actin meshwork is formed through ARP2/3-mediated nucleation of actin filaments (Pollard and Borisy, 2003). This process is controlled by either chemoattractants, giving rise to directional migration, or chemokinetic agents, giving rise to non-directed migration. The formation of new actin meshworks subsequently facilitate the establishment of new focal adhesion contacts (Gupton and Waterman-Storer, 2006; Giannone et al., 2007).

What mechanism pushes the membrane forward is not clear to us. The observation that cells flatten through contraction of actin arcs indicates that protrusion may in part be by a simple consequence of redistribution of the cytoplasm. If the volume of the cytoplasm does not diminish, the cell simply will have to spread (due to hydrostatic pressure). The newly formed actin meshwork may then just serve to fill up the space and prevent a complete rearward movement of the leading edge. The other possibility is that the newly formed actin network plays a role in actively pushing the membrane forward.

Web resources

- Cell Migration Consortium, University of Virginia, http://www.cellmigration.org/topics/retraction.shtml
- The cytoskeleton milestones time line from 1942 to 2000 by Nature Publishing Group, http://www.nature.com/milestones/milecyto/timeline.html

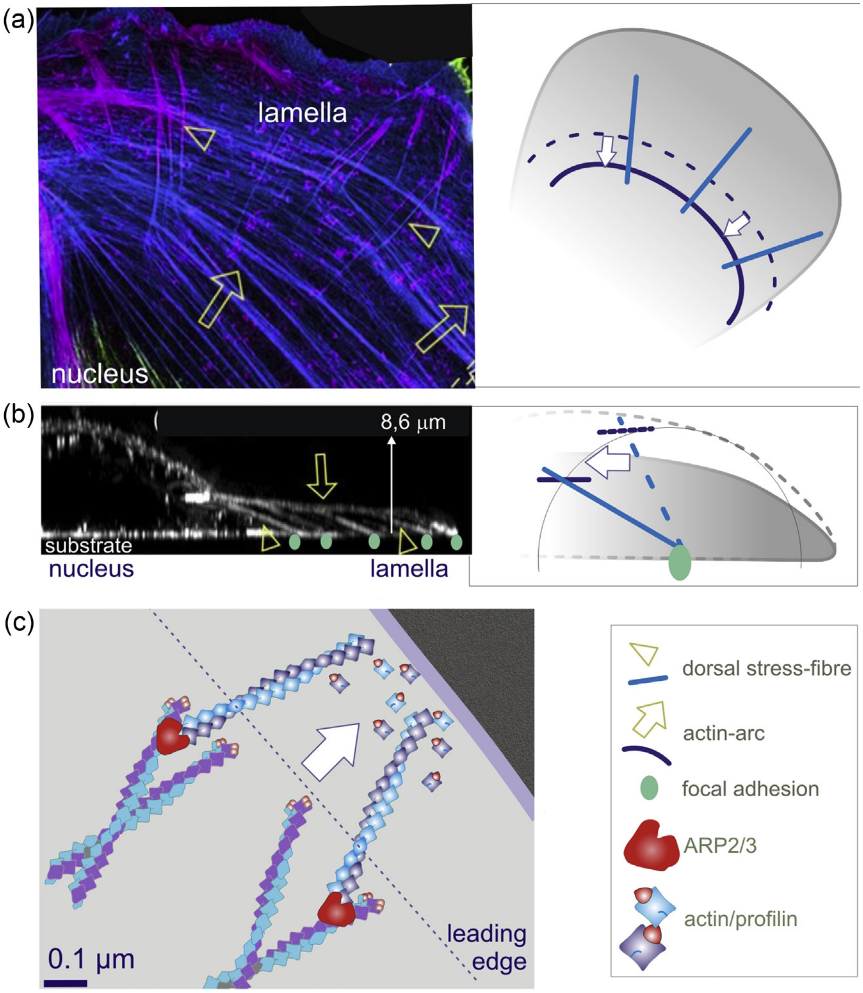


FIGURE 6-22 Roles of actin in cell migration. (a) Actin staining, top view, shows the actin arcs and the perpendicularly associated dorsal stress fibers. Contraction of the actin arcs causes a backward movement of the dorsal tips of the stress fibers. (b) Actin staining, side view, illustrating the position of the actin arcs and the dorsal stress fibers. Note that the latter connect actin arcs with focal adhesion structures (site of attachment to the substrate). Backward movement of the dorsal tips of the stress fibers is only possible when the actin arcs and the stress fibers lower their position (relative to the fixed focal adhesion). As a consequence the lamella flattens. (c) Polymerization of actin at the ARP2/3 nucleation sites creates a new meshwork at the leading edge of the migrating cell. *Images (a) and (b) were obtained with permission from Burnette et al. (2014)*

Connecting Ca^{2+} waves with cytoskeletal activity

Whereas the global intracellular free [Ca^{2+}] is generally more elevated at the tail than is the leading edge in migrating cells, brief Ca^{2+} pulses, lasting in the order of seconds, are observed exclusively near protrusions. When cells follow a chemotactic gradient, the Ca^{2+} pulses accentuate in the portion of the leading edge that faces the source of the chemotactic agent (Figure 6-23). The Ca^{2+} pulses are a consequence of receptor-mediated production of IP_3 and subsequent opening of ITPR2 channels, and may be supported by changes in the permeability of a stretch-activated plasma channel such as TRPM7 (Wei et al., 2009). The repeated Ca^{2+} pulses, set against a very low background, play a role in the above described activities of the actin cytoskeleton. Ca^{2+} influences the events at two levels: (1) by activation of myosin IIA and (2) by promoting the nucleation of actin filaments at the actin-related protein-2 and -3 complexes (ARP2/3). Chemotactic agents, such as platelet-derived growth factor (PDGF), fibroblast-growth factor (FGF), or interleukin-8 (IL8) cause local activation of two effector enzymes: phospholipase Cγ (PLCγ or PLCG1) and phosphatidylinositol 3-kinase (PI 3-K or PIK3C) (Tsai et al., 2014). Activation of phospholipase Cγ gives rise to the production of diacylglycerol and inositol-1,4,5-phosphate (IP_3) and subsequent liberation of Ca^{2+} from internal stores. An important downstream effector is protein kinase Cα (PRKCA). It phosphorylates RhoGDI (on ser-96 in mouse) and this causes a reduction in the affinity for the monomeric GTPases Rac1 and RhoA (Sabbatini and Williams, 2013). RhoGDI (ARHGDIA) is named so, Rho GDP-dissociation inhibitor, because its binding to the monomeric GTPase prevents access of the nucleotide exchange factor; GDP cannot be exchanged for GTP and this keeps the GTPase in an “inactive” state. Liberation and subsequent attachment to the plasma membrane of Rac1 and RhoA results in the encounter with their respective nucleotide exchange factors (Tiam1 and p115RhoGEF) (Figure 6-23). With respect to Rac1, the chemokine-mediated activation of PI3 kinase causes the formation of phosphatidyl-3,4,5-phosphate (PIP_3) which brings the nucleotide exchange factor Tiam1 to the membrane (via its N-terminal PH domain) and results in nucleotide exchange of Rac1 (Weiner et al., 2002; Price et al., 2003). The GTPases Rac1, in concert with Cdc42, then activates a complex of proteins, WAVE/SCAR/WASP, (Takenawa and Miki, 2001) which causes binding of ARP2/3 to existing actin filaments and leads to the initiation of actin-filament formation (nucleation). With respect to RhoA, it activates ROCK1, a protein kinase that phosphorylates the regulatory subunits of the serine/threonine phosphatase PPP1CA which causes its inactivation. Persistent Ca^{2+}-mediated activation of myosin-light chain kinase is the consequence of all this

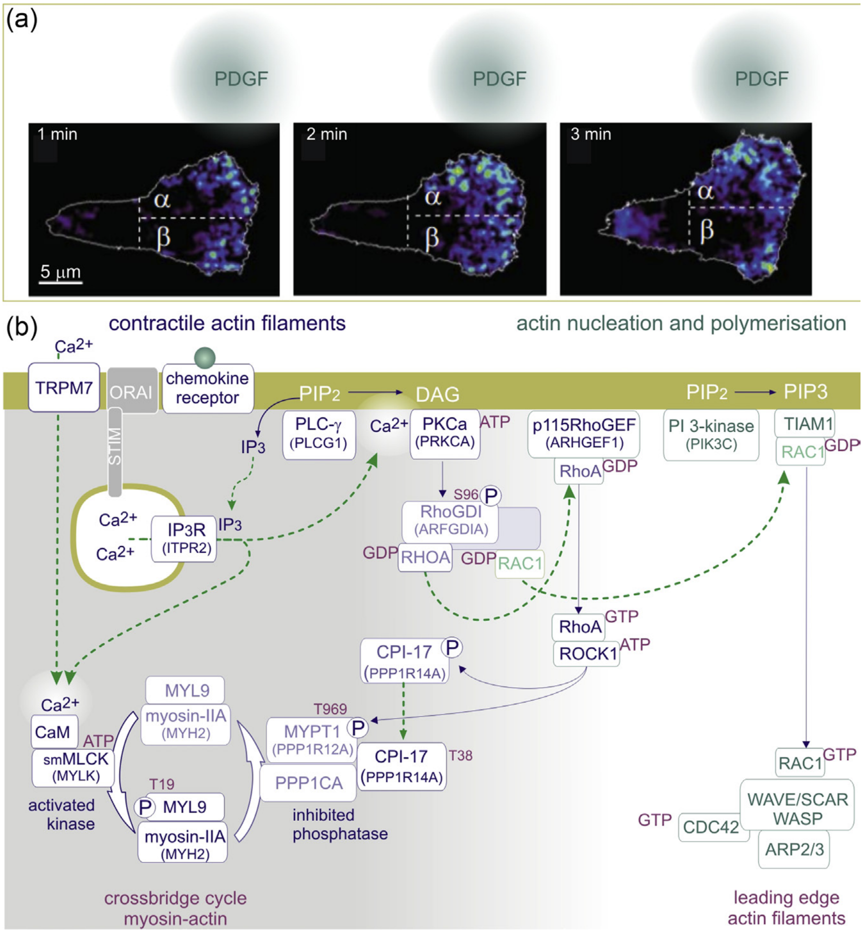


FIGURE 6-23 **Ca^{2+}-pulses steer the migration process.** (a) Calcium pulses (blue/yellow) occur at the leading edge and are particularly abundant at the site near the chemokine source (PDGF in the example) where they steer cell turning (time intervals of 1 min). Image obtained with permission from Wei et al., 2009. (b) Chemokine receptors essentially bring about two processes: one through activation of phospholipase-C (PLC) and leading to shortening of contractile actin filaments, and the other through activation of PI3-kinase) and leading to the formation of a new actin meshwork at the leading edge. The sequence of events is described in the text.

and extensive myosin IIA (MYH2) activity is the result (return to Chapter 3, Figure 3-28). To make the story complete, in blood-borne cells PLCβ-mediated production of diacylglycerol not only causes activation of protein kinase C, it also brings about activation of yet another monomeric GTPase, Rap1b (RAP1B), which is an essential element in the activation of the integrin adhesion molecules and thus essential in the formation of new focal contacts (Han et al., 2006).

MICHAEL ABERCROMBIE A PIONEER IN CELL MIGRATION

We end the chapter by paying tribute to the works of Michael Abercrombie who is generally considered one of the pioneers in the study of cell migration. He felt encouraged to study biology by an article in the *Times* newspaper, written by Julian Huxley (brother of Aldous Huxley, author of the "Brave new world"), which resumed that "biology was a very good thing." After his studies in Oxford, Michael Abercrombie began his career in 1934 with Chris Waddington at the Strangeways Laboratories in Cambridge. The lab worked on chick blastoderms in search of the vertebrate "Spemann organizer" (subject of the Nobel Prize of 1935). In the second of the two articles that arose from this episode, he drew attention to the finding that the interface between grafted tissue and host (the whole embryo) was modified through a process of cell migration (repositioning of the cells relative to each other) (Abercrombie and Waddington, 1937). In 1938, he obtained a lectureship in the Zoology Department in the University of Birmingham, but unfortunately the lab only had a bench and a sink. The story goes that Abercrombie decided to cash in his pension contributions, and Jane Johnson, his new collaborator and wife, sold her car, enabling them to buy the bare essentials for equipping a lab. Here, and for some time in Oxford, he began to work on the analysis of Schwann-cell behavior in tissue culture. Schwann cells emigrating from pieces of severed peripheral nerve play a role in the regeneration process by producing chemoattractants (mainly nerve growth factor) that stimulate the outgrowth of neuronal sprouts into the lesion. This project was instigated as part of a contribution of scientists to the war effort. He found that Schwann cells ceased to migrate after they had contacted each other (contact inhibition), which explained the reduced activity of these cells in vivo after reuniting the stumps of a severed nerve (Abercrombie and Johnson, 1942). In 1947, he moved to University College London, where, in collaboration with Joan Heaysman, time lapse cinemicrography studies started in earnest and revealed how sarcoma cells can become invasive because of a lack of contact inhibition (Abercrombie and Heaysman, 1954). In 1970, he returned to the Strangeways Laboratory in Cambridge, this time as the director. Subsequently, with Sue Pegrum and Joan Heaysman, their observations on "the locomotion of fibroblasts in culture" led to the new idea that "a cell extends itself over the substratum by means of the assembly of a new surface at its leading edge" and "that the body of the cell is drawn up to the new adhesions by the system of contractile filaments" (phenomenon of protrusion and retraction) (Abercrombie et al., 1970). This lead to the foundation of a still very active field of research, and although much progress is made, the mechanism by which cells control membrane protrusions and retractions and how they control the dynamics of adhesion

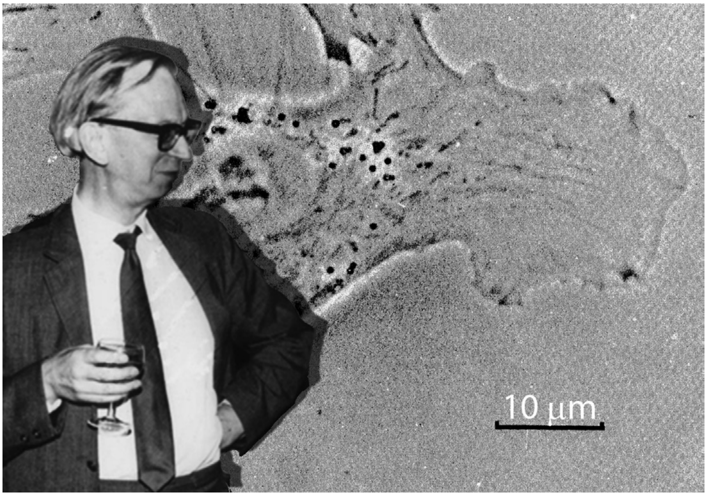


FIGURE 6-24 Michael Abercrombie set against the background of a migrating chick embryo heart fibroblast which he and his colleagues have studied extensively. Image of Michael Abercrombie courtesy of Graham Dunn, Kings College London.

contacts still requires new insights in order to be fully understood (Figure 6-24). In the words of Abercrombie: "*Crawling is one of the most important cell activities in the animal kingdom, important in its generality and in its function. It is just beginning to come into perspective. I make no excuses for such a general lecture, therefore, because it seems to me that comprehensiveness is what is wanted in the subject, which is still inclined to suffer from fragmentation. Of course, at this stage no general picture can be satisfactory. But to generate dissatisfaction is perhaps the main purpose of a general picture, and I am sure I have succeeded in that*" (Abercrombie, 1980).

We obtained biographical information from Dr Michelle Peckham, University of Leeds, UK. For more information about Abercrombie, read Dunn and Jones (1998) and Bellairs (2000).

References

Abercrombie, M., Heaysman, J.E.M., 1954. Nature 174, 697–698.

Abercrombie, M., Johnson, M.L., 1942. The outwandering of cells in tissue cultures of nerves undergoing Wallerrian degeneration. J. Exp. Biol. 266–283.

Abercrombie, M., Waddington, C.H., 1937. The behavior of grafts of primitive streak beneath the primitive streak of the chick. J. Exp. Biol. 14, 319–334.

Abercrombie, M., Heaysman, J.E., Pegrum, S.M., 1970. The locomotion of fibroblasts in culture. 1. Movement of the leading edge. Exp. Cell Res. 59, 393–398.

Abercrombie, M., 1980. The Croonian Lecture, 1978: the crawling movement of metazoan cells. Proc. R. Soc. Lond. Biol. Sci. 207, 129–147.

Ames, J.B., Lim, S., Ikura, M., 2012. Molecular structure and target recognition of neuronal calcium sensor proteins. Front. Mol. Neurosci. 5, 10.

Arif, S.H., 2009. A Ca^{2+} binding protein with numerous roles and uses: parvalbumin in molecular biology and physiology. Bioessays 31, 410–421.

Ashley, C.C., 1969. Aequorin-monitored calcium transients in single Maia muscle fibres. J. Physiol. 203, 32P–33P.

Atlas, D., 2013. The voltage-gated calcium channel functions as the molecular switch of synaptic transmission. Ann. Rev. Biochem. 82, 607–635.

Baba, Y., Nishida, K., Fujii, Y., Hirano, T., Hikida, M., Kurosaki, T., 2008. Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. Nat. Immunol. 9, 81–88.

Bachnoff, N., Cohen-Kutner, M., Trus, M., Atlas, D., 2013. Intro-membrane signaling between the voltage-gated Ca^{2+}-channel and cysteine residues of syntaxin 1A coordinates synchronous release. Nat. Rep. 3, 1620.

Bayer, K.U., de Koninck, P., Schulman, H., 2002. Alternative splicing modulates the frequency-dependent response of CaMKII to Ca^{2+}-oscillations. EMBO J. 21, 3590–3597.

Bellairs, R., 2000. Michael Abercrombie (1912–1979). Int. J. Dev. Biol. 44, 23–28.

Berridge, M.J., Irvine, R.F., 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315–321.

Beyder, A., 2012. Targeting ion channels for the treatment of gastrointestinal motility disorders. Ther. Adv. Gastroentrol. 5, 5–21.

Boitier, E., Rea, R., Duchen, M.R., 1999. Mitochondria exert a negative feedback on the propagation of fintracellular Ca^{2+} waves in rat cortical astrocytes. J. Cell Biol. 145, 795–808.

Bouter, A., Gounou, C., Bérat, R., Tan, S., Gallois, B., Granier, T., d'Estaintot, B.L., Pöschl, E., Brachvogel, B., Brisson, A.R., 2011. Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair. Nat. Commun. 2, 270.

Buckley, R., 2003. Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. Annu. Rev. Immunol. 22, 625–655.

Burnette, D.T., Shao, L., Ott, C., Pasapera, A.M., Fischer, R.S., Baird, M.A., Der Loughian, C., Delanoe-Ayari, H., Paszek, M.J., Davidson, M.W., Betzig, E., Lippincott-Schwartz, J., 2014. A contractile and counterbalancing adhesion system controls the 3D shape of crawling cells. J. Cell Biol. 205, 83–96.

Burtnick, L.D., Urosev, D., Irobi, E., Narayan, K., Robinson, R.C., 2004. Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. EMBO J. 23, 2713–2722.

Calcraft, P.J., Ruas, M., Pan, Z., et al., 2009. NAADP mobilizes calcium from acidic organelles through two-pore channels? Nature 459, 596–600.

Campbell, A.K., 1974. Extraction, partial purification and properties of the Ca^{2+}-activated luminescent protein obelin from the hydroid *Obelia geniculate*. Biochem. J. 143, 411–481.

Capes, E.M., Loaiza, R., Valdivia, H.H., 2011. Ryanodine receptors. Skelet. Muscle 1, 18–30.

Carrithers, M.D., Chatterjee, G., Carrithers, L.M., Offoha, R., Lheagwara, U., Rahner, C., Graham, M., Waxman, S.G., 2009. Regulation of podosome formation in macrophages by a splice variant of the sodium channel SCN8A. J. Biol. Chem. 284, 8114–8126.

Chao, L.H., Stratton, M.M., Lee, I.-H., Rosenberg, O.S., Levitz, J., Mandell, D.K., Kortemme, T., Groves, J.T., Sculman, H., Kuriyan, J., 2011. A mechanism for tunable autoinhibition in the structure of a human Ca^{2+}/Calmodulin-dependent kinase II holoenzyme. Cell 146, 732–745.

Chapman, E.R., 2008. How does synaptotagmin trigger neurotransmitter release? Ann. Rev. Biochem. 77, 615–641.

Chen, T.-W., Wardill, T.J., Sun, Y., Pulver, S.R., REnninger, S.L., Baohan, A., Schrrieter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., Looger, L.L., Svoboda, K., Kim, D.S., 2013. Ultrasensitive florescent proteins for imaging neuronal activity. Nature 499, 295–300.

Cheung, W.Y., 1970. Cyclic 3′,5′-nucleotide phosphodiesterase, demonstration of an activator. Biochem. Biophys. Res. Commun. 38, 533–538.

Coe, H., Michalak, M., 2009. Calcium binding chaperones of the endoplasmic reticulum. Gen. Physiol. Biophys. 28, F96–F103.

Corbett, E.F., Michalak, M., 2000. Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem. Sci. 25, 307–311.

Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U., Ullrich, A., 1986. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 233, 859–866.

Creutz, C.E., Pazoles, C.J., Pollard, H.B., 1978. Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of chromaffin granules. J. Biol. Chem. 253, 2858–2866.

D'Acquisto, F., Perretti, M., Flower, R.J., 2008. Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. Br. J. Pharmacol. 155, 152–169.

Dolmetsch, R., Xu, K., Lewis, R., 1998. Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392, 933–938.

Duchen, M.R., 2000. Mitochondria and calcium: from cell signalling to cell death. J. Physiol. 529, 57–68.

Dunn, G., Jones, G., 1998. Michael Abercrombie: the pioneer ethologist of cells. Trends Cell Biol. 8, 124–126.

Dupont, G., Combettes, L., Leybaert, L., 2007. Calcium dynamics: spatio-temporal organization from the subcellular to the organ level. Int. Rev. Cytol. 261, 193–245.

Ebashi, S., Endo, M., 1968. Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. 18, 123–183.

Ferrier, K., 2012. A Film by Diane Perelsztejn. Ideale audience/Arte France/Decca.

Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S.-H., Tanasa, B., Hogan, P.G., Lewis, R.S., Daly, M., Rao, A., 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441, 179–185.

Filopanti, M., Coretta, S., Barbieri, A.M., Spada, A., 2013. Pharmacology of the calcium sensing receptor. Clin. Cases Miner. Bone Metab. 10, 162–165.

Flower, R.J., Blackwell, G.J., 1979. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. Nature 278, 456–459.

Flynn, E.R., Bradley, K.N., Muir, T.C., McCarron, J.H., 2001. Functionally separated intracellular Ca^{2+} stores in smooth muscle. J. Biol. Chem. 276, 36411–36418.

Fraser, S., Salvador, V., Manning, E., Mizal, J., Altun, S., Raza, M., Berridge, R., Djamgoz, M., 2003. Contribution of functional voltage-gated Na^{+} channel expression to cell behaviors involved in the metastatic cascade in rat prostate cancer: I. lateral motility. J. Cell Physiol. 195, 479–487.

Galione, a, Lee, H.C., Busa, W.B., 1991. Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. Science 253, 1143–1146.

Gardner, P., 1989. Calcium and T lymphocytes. Cell 59, 15–20.

Gerke, V., Creutz, C.E., Moss, S.E., 2005. Annexins: linking Ca^{2+} signalling to membrane dynamics? Nat. Rev. Mol. Cell Biol. 6, 449–460.

Ghosh, T.K., Bian, J., Gill, D., 1994. Sphingosine-1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. J. Biol. Chem. 269, 22628–22635.

Giannone, G., Dubin-Thaler, B.J., Rossier, O., Cai, Y., Chaga, O., Jiang, G., Beaver, W., Dobereiner, H.-G., Freund, Y., Borisy, G., Sheetz, M.P., 2007. Lamellipoidal actin mechanically links myosin activity with adhesion-site formation. Cell 128, 561–575.

Grill, V., Ho, P., Body, J.J., Johanson, N., Lee, S.C., Kukreja, S.C., Moseley, J.M., Martin, T.J., 1991. Parathyroid hormone-related protein: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. J. Clin. Endocrinol. Metab. 73, 1309–1315.

Grynkieuwicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. J. Biol. Chem. 260, 3440–3450.

Gupton, S.L., Waterman-Storer, C.M., 2006. spatiotemporal feedback between actinomyosin and focal-adhesion systems optimizes rapid cell migration. Cell 125, 1361–1374.

Guse, A.H., da Silva, C.P., Berg, I., et al., 1999. Regulation off calcium signalling in T lymphocytes by the second messenger cycl ADP-ribose. Nature 398, 70–73.

Guse, A.H., 2004. Regulation of calcium signaling by the second messenger cyclic adenosine diphosphoribose cADPR. Curr. Mol. Med. 4, 239–248.

Hajnoczky, G., Thomas, A., 1997. Minimal requirements for calcium oscillations driven by the IP_3 receptor. EMBO J. 16, 3533–3543.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J., Ginsberg, M.H., 2006. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIb-beta3. Curr. Biol. 16, 1796–1806.

Hannun, Y.A., Obeid, L.M., 2008. Principles of bioactive lipid signalling: lessons from spingophospholipids. Nat. Rev. Mol. Cell Biol. 9, 139–150.

Harvey, A.M., MacIntosh, F.C., 1940. Calcium and synaptic transmission in a sympathetic ganglion. J. Physiol. 97, 408–416.

Heilbrunn, L.V., Wiercinsky, F.J., 1947. The action of various cations on muscle protoplasm. J. Cell Comp. Physiol. 29, 15–23.

Heilbrunn, L.V., 1940. The action of calcium on muscle protoplasm. Physiol. Zool. 13, 88–94.

Heizmann, C.W., Ackermann, G.E., Galichet, A., 2007. Pathologies involving the S100 proteins and RAGE. Subcell. Biochem. 45, 93–138.

Hilge, M., Aelen, J., Vuister, G.W., 2006. Ca^{2+}-regulation in the Na^+/Ca^{2+} exchanger involves two markedly different Ca2+ sensors. Mol. Cell 22, 15–25.

Hinigmann, A., van den Bogaart, G., Irahet, E., Risselada, J.H., Milovanovic, D., Mueller, V., Mullar, S., Diederichsen, U., Fasshauer, D., Grubmuller, H., Hell, S.W., Eggeling, C., Kuhnel, K., Jahn, R., 2013. Phosphtidylinositol 4,5,-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nat. Struct. Mol. Biol. 20, 679–686.

Hoeflich, K.P., Ikura, M., 2002. Calmodulin in action: diversity in target recognition and activation mechanisms. Cell 108, 739–742.

Hoelz, A., Nairn, A.C., Kuriyan, J., 2003. Crystal structure of a tetradecameric assembly of the association domain of Ca^{2+}/calmodulin-dependent kinase II. Mol. Cell 11, 1241–1251.

Hofmann, T., Obukhov, A.G., Schaefer, M., Harteneck, C., Gudermann, T., Schultz, G., 1999. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397, 259–263.

Hoque, M., Rentero, C., Cairns, R., Tebar, F., Enrich, C., Grewal, T., 2014. Annexins- Scaffolds modulating PKC localization and signaling. Cell Signal 26, 1213–1225.

Hoth, M., Penner, R., 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature. 355, 353–356. http://www.ncbi.nlm.nih.gov.gate2.inist.fr/pubmed/1309940.

Hou, X., Pedi, L., Diver, M.D., Long, S.B., 2012. Crystal structure of the calcium release-activated calcium channel Orai. Science 338, 1308–1313.

Houdusse, A., Cohen, C., 1996. Structure of the regulatory domain of scallop myosin at 2 A resolution: implications for regulation. Structure 4, 21–32.

Hu, J., Spiegel, A.M., 2007. Structure and function of the human calcium-sensing receptor; insights from natural and engineered mutations and allosteric modulators. J. Cell Mol. Med. 11, 908–922.

Hu, X.-W., Zhang, L., 2012. Function and regulation of large conductance Ca^{2+}-activated K^+ channel in vascular smooth muscle cells. Drug Discov. Today 17, 974–987.

Huang, Y., Zhou, Y., Yang, W., Butters, R., Lee, H.W., Li, S., Castiblanco, A., Brown, E.M., Yang, J.J., 2007. Identification and dissection of Ca^{2+}-binding sites in the extracellular domain of Ca^{2+}-sensing receptor. J. Biol. Chem. 282, 19000–19010.

Imagawa, T., Smith, J.S., Coronado, R., Campbell, K.P., 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore off the calcium release channel. J. Biol. Chem. 262, 16636–16643.

Jacob, R., Merritt, J.E., Hallam, T.J., Rink, T.J., 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. Nature 335, 1153–1159.

Johnson, R.W., Nguyen, M.P., Padalecki, S.S., Grubbs, B.G., Merkel, A.R., Oyajobi, B.O., Matrisian, L.M., Mundy, G.R., Sterling, J.A., 2011. TGF-beta promotion of Gli2-induced expression of parathyroid hormone-related protein, an important osteolytic factor in bone metastasis, is independent of canonical Hedgehog signaling. Cancer Res. 71, 822–831.

Kaeser, P.S., et al., 2011. RIM proteins thether Ca^{2+} channels to presynaptic active zones via a direct PDZ-domain interaction. Cell 144, 282–295.

Kakiuchi, S., Yamazaki, R., 1970. Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain studies on cyclic 3′,5′-nucleotide phosphodiesterase. Biochem. Biophys. Res. Commun. 41, 1104–1110.

Kamada, T., Kinoshita, H., 1943. Disturbances initiated from naked surface of muscle protoplasm. Jpn. J. Physiol. 10, 469–493.

Karakozova, M., Kozak, M., Wong, C.C.L., Bailey, A.O., Yates, J.R., Mogilner, A., Zebroski, H., Kashina, A., 2006. Arginylation of β-actin regulates actin cytoskeleton and cell motility. Science 313, 192–196.

Kasai, H., Augustine, G., 1990. Cytosolic Ca^{2+} gradients triggering unidirectional fluid secretion from exocrine pancreas. Nature 348, 735–738.

Kato, I., Yamamoto, Y., Fujimura, M., Noguchi, N., Takasawa, S., Okamoto, H., 1999. CD38 disruption impairs glucose-induced increases in cyclic ADP-ribose, $[Ca^{2+}]i$ and insulin secretion. J. Biol. Chem. 274, 1869–1872.

Katz, B., Miledi, R., 1969a. Tetrodotoxin-resistannt electric activity in presynaptic terminals. J. Physiol. 203, 459–487.

Katz, B., Miledi, R., 1969b. Spontaneous and evoked activity off motor nerve endings in calcium Ringer. J. Physiol. 203, 689–706.

Katz, B., 1970. On the Quantal Mechanism of Neural Transmitter Release. Nobel Lecture.

Kimlicka, L., Tung, C.-C., Carlsson, A.C.C., Lobo, P.A., Yuchi, Z., van Petegem, F., 2013a. The cardiac ryanodine receptor N-terminal region contains an anion binding site that is targeted by disease mutations. Structure 21, 1440–1449.

Kimlicka, K., Lau, K., Tung, C.C., van Petegem, F., 2013b. Disease mutations in the ryanodine receptor N-terminal region couple to a mobile intersubunit interface. Nat. Commun. 4, 1506.

Kirichok, Y., Krapivinsky, G., Clapham, D.E., 2003. The mitochondrial calcium uniporter is a highly selective ion channel. Nature 427, 360–364.

Kuma, S., Ahmad, E., Kumar, S., Khan, R.H., Gourinath, S., 2012. Flexibility of EF-hand motifs: structural and thermodynamic studies off calcium binding protein-1 from *Entamoeba histolytica* with Pb^{2+}, Ba^{2+} and Sr^{2+}. BMC Biophys. 5, 15.

Kuno, M., Goronxy, J., Weyand, C.M., Gardner, P., 1986. Single-channel and whole-cell recordings of mitogen-regulated inward currents in human cloned helper T lymphocytes. Nature 323, 269–273.

Lee, H.C., Aarhus, R., 1995. A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. J. Biol. Chem. 270, 2152–2157.

Lee, H.C., 2006. Structure and enzymatic functions of human CD38. Mol. Med. 12, 317–328.

Llinas, R., Blinks, J.R., Nicholson, 1972. Calcium transients in presynaptic terminal of squid giant synapse: detection with aequorin. Science 176, 1127–1129.

Locke, F.S., 1894. Notiz über den einfluss der physiologischer kochsalzlösung auf di erregbarkeit von muskel und nerv. Zentralbl. Physiol. 8, 166–167.

Ludtke, S.J., Serysheva II, Hamilton, S.L., Chiu, W., 2005. The pore structure off the closed RYR1 channel. Structure 13, 1203–1211.

Oh-Hora, M., Yamashita, M., Hogan, P.G., Sharma, S., Lamperti, E., Chung, W., Prakriya, M., Feske, S., Rao, A., 2008. Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. Nat. Immunol. 9, 432–443.

Mak, D.O., McBride, S., Foskett, J.K., 1998. Inositol-1,4,5-trisphosphate activation of inositol tris-phosphate receptor Ca^{2+} channel by ligand tuning of Ca^{2+} inhibition. Proc. Natl. Acad. Sci. 95, 15821–15825.

Mak, D.O., McBride, S., Ragharum, V., Yue, Y., Joseph, S.K., Foskett, J.K., 2000. Single channel properties in endoplasmic reticulum membrane of recombinant type 3 inositol tris phosphate receptor. J. Gen. Physiol. 4, 239–248.

Malenka, R.C., Bear, M.F., 2004. LTP and LTD: and embarrassment of riches. Neuron 44, 5–21.

Mann, P.J.G., Tennenbaum, M., Quastel, J.H., 1939. Acetylcholine metabolism in the central nervous systel: the effects of potassium and other cations on acetylcholine liberation. Biochem. J. 33, 822–835.

Merrit, J.E., Rink, T.J., 1987. Rapid increases in cytosolic free calcium in response to muscarinic stimulation of rat parotid acinar cells. J. Biol. Chem. 262, 4958–4960.

Merritt, J.E., Rink, T.J., 1987. Rapid increases in cytosolic free calcium in response to muscarinic stimulation of rat parotid acinar cells. J. Biol. Chem. 262, 4958–4960.

Meyer-zu-Heringdorf, D., Liliom, K., Schaefer, M., et al., 2003. Photolysis of intracellular caged sphingosine-1-phosphate causes Ca^{2+} mobilization independently of G-protein coupled receptors. FEBS Lett. 554, 443–449.

Michell, R.H., 1975. Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 81–147.

Montell, C., 2005. TRP channels in Drosophila photoreceptor cells. J. Physiol. 567, 45–51.

Mosca, B., Delbono, O., Laura Messi, M., Bergamelli, L., Wang, Z.M., Vukcevic, M., Lopez, R., Treves, S., Nishi, M., Takeshima, H., Paolini, C., Martini, M., Rispoli, G., Protasi, F., Zorzato, F., 2013. Enhanced dihydropyridine receptor calcium channel activity restores muscle strength in JP45/CASQ1double knockout mice. Nat. Commun. 4, 1541.

Mountford, J.C., Melford, S.K., Bunce, C.M., Gibbins, J., Watson, S.P., 1999. Collagen or collagen-related peptide cause Ca^{2+} elevation and increased tyrosine phosphorylation in human megakaryocytes. Thromb. Haemost. 82, 1153–1159.

Mujica, P.E., González, F.G., 2011. Interaction between IP_3 receptors and BK channels in arterial smooth muscle: non-canonical IP_3 signaling at work. J. Gen. Physiol. 137, 473–477.

Nakai, J., Ohkura, M., Imoto, K., 2001. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. Nat. Biotechnol. 19, 137–141.

Nakashima, Y., Nishimura, S., Maeda, A., Barsoumian, E.L., Hakamata, Y., Naki, J., allen, P.D., Imoto, K., Kita, T., 1997. Molecular cloning and characterization of a human brain ryanodine receptor. FEBS 157–162.

Narayanan, D., Adebiyi, A., Jaggar, J.H., 2012. Inositol trisphosphate receptors in smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol. 302, H2190–H2210.

O'Day, D.H., 2003. CaMBOT: profiling and characterizing calmodulin-binding proteins. Cell Signal. 15, 347–354.

Oritani, K., Kincade, P.W., 1996. Identification of stromal cell products that interact with pre-B cells. J. Cell Biol. 134, 771–782.

Parente, L., Solito, E., 2004. Annexin 1: more than an anti-phospholipase protein. Inflamm. Res. 53, 125–132.

Parker, N.J., Begley, C.C., Smith, P.J., Fox, R.M., 1996. Molecular cloning of a novel human gene (DS11S4896E) at chromosomal region 11p15.5. Genomics 37, 253–256.

Peinelt, C., Vig, M., Koomoa, D.L., Beck, A., Nadler, M.J.S., Koblan-Huberson, M., Lis, A., Fleig, A., Penner, R., Kinet, J.-P., 2006. Amplification of CRAC current by STIM1 and CRACM1 (Orai1). Nat. Cell Biol. 8, 771–773.

Perez, J.F., Sanderson, M.J., 2005. The frequency of calcium oscillations induced by 5-H, ACh and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. J. Gen. Physiol. 125, 535–553.

Perin, M.S., Fried, V.A., Mignery, G.A., Jahn, R., Sudhof, T.C., 1990. Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature 345, 260–263.

Pertill, A., De Carvalho, C.L., Matsumura, C.Y., 2010. Calcium-binding protein in skeletal muscles of the mdx mice: potential role in the pathogenesis of Duchenne muscular dystrophy. Int. J. Exp. Pathol. 91, 63–71.

Pollack, H., 1928. Micrurgical studies in cell physiology: VI calcium ions in living protoplasm. J. Gen. Physiol. 11, 539–545.

Pollak, M.R., Brown, E.M., Chou Y-H, W., Hebert, S.C., Marx, S.J., Steinmann, B., Levi, T., Seidman, C.E., Seidman, J.G., 1993. Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. Cell 75, 1297–1303.

Pollard, T.D., Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–465.

Price, L.S., Langslag, M., ten Klooster, J.P., Hordijk, P.L., Jalink, K., Collard, J.G., 2003. Calcium signaling regulates translocation and activation of Rac. J. Biol. Chem. 278, 39413–39421.

Rebbeck, R.T., Karunasekara, Y., Board, P.G., Beard, N.A., Casarotto, M.G., Dulhunty, A.F., 2014. Skeletal muscle excitation-contraction coupling: who are the dancing partners? Int. J. Biochem. Cell Biol. 48, 28–38.

Reichow, S.L., Clemens, D.M., Freites, J.A., Németh-Cahalan, K.L., Heyden, M., Tobias, D.J., Hall, J.E., Gonen, T., 2013. Allosteric mechanism of water-channel gating by Ca^{2+}-calmodulin. Nat. Struct. Mol. Biol. 20, 1085–1095.

Rhee, S.G., 2001. Regulation of phosphoinositide-specific phospholipase C. Ann. Rev. Biochem. 70, 281–312.

Ridgeway, E.B., Ashley, C.C., 1967. Calcium transients in single muscle fibers. Biochem. Biophys. Res. Com. 29, 229–234.

Ringer, S., 1883. Regarding the action of the hydrate of soda, hydrate of ammonia and hydrate of potash on the ventricle of the frog's heart. J. Physiol. 3, 195–202.

Ringer, S., 1884. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. 4, 29–42.

Sabbatini, M., Williams, J.A., 2013. Cholecystokinin-mediated RhoGDI phosphorylation via PKCa promotes both RhoA ad Rac1 signaling. PLoS One 8, e66029.

Sabbioni, S., Barbanti-Brodano, G., Croce, C.M., Negrini, M., 1997. GOK: a gene at 11p15 involved in rhabdomyosarcoma and rhabdoid tumor development. Cancer Res. 57, 4493–4497.

Sato, C., Hamada, K., Ogura, T., Miyazawa, A., Iwasaki, K., Hiroaki, Y., Tani, K., Terauchi, A., Fujiyoshi, Y., Mikoshiba, K., 2004. Inositol 1,4,5-trisphosphate receptor contains multiple cavities and L-shaped ligand-binding domains. J. Mol. Biol. 336, 155–164.

Schumacher, M.A., Rivard, A.F., BAchinger, H.P., Adelman, J.P., 2001. Crystal structure of the gating domain from small conductance potassium channel complexed with calcium-calmodulin. Nature 410, 1120–1124.

Seo, M.-D., Velamakanni, S., Ishiyama, N., Sathopulos, P.B., Rossi, A.M., Khan, S.A., Dale, P., Li, C., Ames, J.B., Ikura, M., Taylor, C.W., 2010. Structural and functional conservation of key domains in InsP3 and ryanodine receptors. Nature 483, 108–112.

Shimomura, O., Johnson, F.H., 1969. Properties of the biomuminescent protein aequorin. Biochemistry 8, 3991–3997.

Shimomura, O., Johnson, F.H., Saiga, Y., 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedsan Aequorea. J. Cell Comp. Physiol. 59, 223–239.

Silva, A.J., Stevens, C.F., Tonegawa, S., Wang, Y., 1992a. Deficient hippocampal long-term potentiation in a-calcium-calmodulin kinase II mutant mice. Science 257 (5067), 201–206.

Silva, A.J., Paylor, R., Wehner, J.M., Tonegawa, S., 1992b. Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257 (5067), 206–211.

Sneyd, J., Tsaneva-Atanasova, K., Reznikov, V., Bai, Y., Sanderson, M.J., Yule, D.I., 2006. A method for determining the dependence of calcium oscillations on inositol trisphosphate oscillations. Proc. Natl. Acad. Sci. U.S.A. 103, 1675–1680.

Soboloff, J., Spassova, M.A., Tang, X.D., Hewavitharana, T., Xu, W., Gill, D.L., 2006. Orai1 and STIM reconstitute store-operated calcium channel function. J. Biol. Chem. 281, 20661–20665.

Solomon, J.P., Page, L.J., Balch, W.E., Kelly, J.W., 2012. Gelsolin amyloidosis: genetics, biochemistry, pathology and possible strategies for therapeutic intervention. Crit. Rev. Biochem. Mol. Biol. 47, 282–296.

Spiegel, S., Milstien, S., 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell Biol. 4, 397–407.

Stevens, C.F., Sullivan, J.M., 2003. The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission. Neuron 39, 299–308.

Stiber, J., Hawkins, A., Zhang, Z.-S., Wang, S., Burch, J., Graham, V., Ward, C.C., Seth, M., Finch, E., Malouf, N., Williams, R.S., Eu, J.P., Rosenberg, P., 2008. STIM1 signalling controls store operated calcium entry required for the development and contractile function in skeletal muscle. Nat. Cell Biol. 10, 688–697.

Streb, H., Irvine, R.F., Berridge, M.J., Schulz, I., 1983. Release of Ca^{2+} from a nonmitochondrial intracelljlar store in pancreatic acinar cells by inositol-1,4,5-trisphospate. Nature 306, 67–69.

Sudhof, T.C., Rothman, J.E., 2009. Membrane fusion : grapling with SNARE and SM proteins. Science 323, 474–477.

Sudhof, T.C., 2013. A molecular machine for neurotransmitter release: synaptotagmin and beyond. Nat. Med. 19, 1227–1231.

Sutherland, E.W., 1972. Studies on the mechanism of hormone action. Science 177, 401–408.

Takenawa, T., Miki, H., 2001. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J. Cell Sci. 114, 1801–1809.

Takeuchi, A., Kim, B., Matsuoka, S., 2013. The mitochondrial Na^{+}-Ca^{2+} exchanger, NCLX, regulates automaticity off HL-1 cardiomyocytes. Nat. Sci. Rep. 3, 2766.

Taylor, C.W., da Fonseca, P.C.A., Morris, E.P., 2004. IP3 receptors: the search for structure. Trends Biochem. Sci. 29, 210–219.

Taylor, C.W., Torey, S.C., Rossi, A.M., Lopez Sanjurjo, C.I., Prole, D.L., Rahman, T., 2014. Structural organization of signalling to abd from IP3 receptors. Biochem. Soc. Trans. 42, 63–70.

Tebar, F., Gelabert-Baldrich, M., Hoque, M., Cairns, R., Rentero, C., Pol, A., Grewal, T., Enrich, C., 2014. Annexins and endosomal signaling. Methods Enzym. 535, 55–73.

Terashima, H., Picollo, A., Accardi, A., 2013. Purified TMEM16A is sufficient to form Ca^{2+}-activated Cl^{-} channels. Proc. Natl. Acad. Sci. U.S.A. 110, 19354–19359.

Thurley, K., Skupin, A., Thul, R., Falcke, M., 2012. Fundamental properties of Ca^{2+} signals. Biochim. Biophys. Acta 1820, 1185–1194.

Tomás, M., Vázquez, E., Fernández-Fernández, J.M., Subirana, I., Plata, C., Heras, M., Vila, J., Marrugat, J., Valverde, M.A., Sentí, M.J., 2008. Genetic variation in the KCNMA1 potassium channel alpha subunit as risk factor for severe essential hypertension and myocardial infarction. J. Hypertens. 26, 2147–2153.

Tombes, R.M., Faison, M.O., Turbeville, J.M., 2003. Organization and evolution of multifunctional Ca^{2+}/CaM-dependent protein kinase genes. Gene 322, 17–31.

Toyoshima, C., 2009. How Ca^{2+} ATPase pumps ions across the sarcoplasmic reticulum membrane. Biochim. Biophys. Acta 1793, 941–946.

Tsai, F.-C., Seki, A., Yang, H.W., Hayer, A., Carrrasco, S., Malmersjo, S., Meyer, T., 2014. A polarized Ca^{2+}, diacylglcyerol and STIM1 signalling system regulates directed cell migration. Nat. Cell Biol. 16, 133–144.

Tsien, R.Y., Pozzan, T., Rink, T.J., 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. 94, 325–334.

Tsien, R.Y., 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. Biochemistry 19, 2396–2404.

Tsien, R.Y., 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 290, 527–528.

Tung, C.C., Lobo, P.A., Kimlicka, L., Van Petegem, F., 2010. The amino-terminal disease hotspot of ryanodine receptors forms a cytoplasmic vestibule. Nature 468, 585–588.

Tunwell, R.E.A., Wickenden, C., Bartrand, B.M.A., Shevchenko, V.I., Walsh, M.B., Allen, P.D., Lai, F.A., 1996. The human cardiac muscle ryanondine receptor-calcium release channel: identification, primary structure and topological analysis. Biochem. J. 318, 477–487.

Venturi, E., Pitt, S., Galfre, E., Sitsapesan, R., 2012. From eggs to hearts: what is the link between cyclic ADP-ribose and ryanodine receptors? Cardiovasc. Ther. 30, 109–116.

Vig, M., Peinelt, C., Beck, A., Koomoa, D.L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., Kinet, J.P., 2006. CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science 312, 1220–1223.

Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tiard, R., Sinclaire, L.K., Foeller, C., Chow, E.P., Browning, J.L., Ramachandran, K.L., Pepinsky, R.B., 1986. Cloning and expression of human lipocortin, a phospholipae A2 inhibitor with potential anti-inflammatory activity. Nature 320, 77–78.

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L.-S., Cheng, H., 2009. Calcium flickers steer cell migration. Nature 457, 901–905.

Weiner, O.D., Neilsen, P.O., Prestwich, G.D., Kircher, M.W., 2002. Cantley LC, Bourne HR. A PtdInsP3- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. Nat. Cell Biol. 4, 509–513.

Weng, X., Luecke, H., Song, I.S., Kang, D.S., Kim, S.H., Huber, R., 1993. Crystal structure of human annexin I at 2.5 A resolution. Protein Sci. 2, 448–458.

Yamasaki, M., Thomazs, J.M., Churchill, G.C., et al., 2005. Role of NAADP and cADPR in the induction and maintenance of agonist-evoked Ca^{2+} spiking in mouse pancreatic acinar cells. Curr. Biol. 15, 874–878.

Yap, K.L., Kim, J., Truong, K., Sherman, M., Yuan, T., Ikura, M., 2000. Calmodulin target database. J. Struct. Funct. Genomics 1, 8–14.

Yuan, P., Leonetti, M.D., Pico, A.R., Hsiung, Y., McKinnon, R., 2010. Structure of the human BL channel Ca^{2+} activation apparatus at 3.0 A resolution. Science 329, 182–186.

Zariwala, H., Borghuis, B.G., Hoogland, T.M., Madisen, L., Tian, L., De Zeeuw, C.I., Zeng, H., Looger, L.L., Svoboda, K., Chen, T.-W., 2012. A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. J. Neurosci. 32, 3131–3141.

Zhang, S.L., Yeromin, A.V., Yu, Y., Safrina, O., Penna, A., Roos, J., Stauderman, K.A., Cahalan, M.D., 2006. Genome-wide RNAi screen of Ca^{2+} influx identifies genes that regulate Ca^{2+} release-activated Ca^{2+} channel activity. Proc. Natl. Acad. Sci. U.S.A. 103, 9357–9362.

Zhao, Y.J., Lam, C.M., Lee, H.C., 2012. The membrane-bound enzyme CD38 exists in two opposing orientations. Sci. Signal. 5, 241.

Zhou, Y., Srinivasan, P., Razavi, S., Seymour, S., Meraner, P., Gudlur, A., Stathopulos, P.B., Ikura, M., Rao, A., Hogan, P.G., 2013. Initial activation of STIM1, the regulator off store-operated calcium entry. Nat. Struct. Mol. Biol. 973–981.

Zicha, D., Genot, E., Dunn, G.A., Kramer, I.M., 1999. TGFβ1 induces a cell-cycle dependent increase in motility of epithelial cells. J. Cell Sci. 112, 447–454.

Zimmerman, B., 2000. Subcellular organization of agonist-evoked Ca^{2+}-waves in the blowfly salivary gland. Cell Calcium 27, 297–307.

CHAPTER

7

Bringing the Signal into the Nucleus: Regulation of Gene Expression

The ability to adjust the genetic readout to changing environmental conditions is an essential condition of organisms. All signal transduction pathways starting at the plasma membrane end in the nucleus, one way or another, and impact on gene expression. With genes we refer to coding regions of the genome that give rise to mRNA and, after translation, to proteins (the flow of information which constitutes the "central dogma of molecular biology" as stated by Francis Crick (1970)). However, it is important to notice that in humans, gene expression goes beyond the transcription of mRNA. It also implies the transcription of ribosomal RNA, transfer RNA, small nuclear RNA, small nucleolar RNA, antisense RNA, long noncoding RNA, microRNA, small interfering RNA, piwi-interacting RNA, and enhancer RNA (Figure 7-1). The process of transcription is controlled by transcription factors and these, in turn, are controlled by signal transduction pathways. In this chapter, we will deal with glucagon-mediated control of gluconeogenesis, the metabolic process through which glucose is released in the blood during periods of nutritional deficiency. We focus on protein kinase A (PKA) and the cAMP response element-binding protein (CREB) and how they control the protein-coding transcription machinery.

Web-based resources

- For more information about the number of human genes, we refer to the GENCODE project database www.gencodegenes

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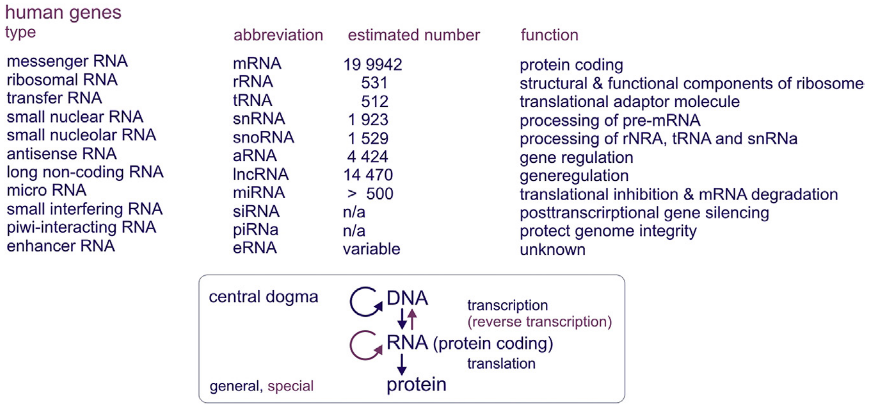


human genes

type	abbreviation	estimated number	function
messenger RNA	mRNA	19 9942	protein coding
ribosomal RNA	rRNA	531	structural & functional components of ribosome
transfer RNA	tRNA	512	translational adaptor molecule
small nuclear RNA	snRNA	1 923	processing of pre-mRNA
small nucleolar RNA	snoRNA	1 529	processing of rNRA, tRNA and snRNa
antisense RNA	aRNA	4 424	gene regulation
long non-coding RNA	lncRNA	14 470	generegulation
micro RNA	miRNA	> 500	translational inhibition & mRNA degradation
small interfering RNA	siRNA	n/a	posttranscrirptional gene silencing
piwi-interacting RNA	piRNa	n/a	protect genome integrity
enhancer RNA	eRNA	variable	unknown

FIGURE 7-1 Human genes and the central dogma of molecular biology. Note that transcription involves much more than transcribing protein-coding genes. *Information from Lee and Young (2013a).*

GLUCONEOGENESIS

Glucose homeostasis in mammals is primarily maintained through glucose uptake in organs in the fed state and production of glucose by the liver during fasting. Immediately after a meal, insulin signals the liver to attenuate glucose production (arresting both glycogenolysis and gluconeogenesis) and instead to focus on glycogenesis. It also signals the muscle and adipose tissue to increase glucose uptake for the same purpose. Conversely, in the fasted state, glucagon signals the liver to arrest glycogenesis and to increase glycogenolysis and at a later stage gluconeogenesis, thus ensuring constant blood glucose levels (avoiding a state of hypoglycemia). Gluconeogenesis is the process that leads to the generation of glucose from a variety of sources such as pyruvate, lactate, glycerol, and certain amino acids. It is largely regulated at the transcriptional level, through enhanced expression of metabolically rate-limiting enzymes. Gluconeogenesis occurs in plants, animals, fungi, and bacteria. In humans, gluconeogenesis occurs in the liver and in the renal cortex in a 3:1 ratio (Gerich et al., 2001). The main gluconeogenic precursors, warranting 90% of glucose production, are lactate (produced during glycolysis), glycerol (produced during breakdown of triglycerides), alanine, and glutamine (produced through breakdown of striated muscle) (Gerich et al., 2001). A brief outline of the metabolic events is shown in Figure 7-2.

GLUCAGON AND GLUCOCORTICOIDS AUGMENT GLUCONEOGENESIS

The main hormone for the regulation of gluconeogenesis is glucagon, a peptide hormone produced by alpha-cells in the endocrine pancreas (in

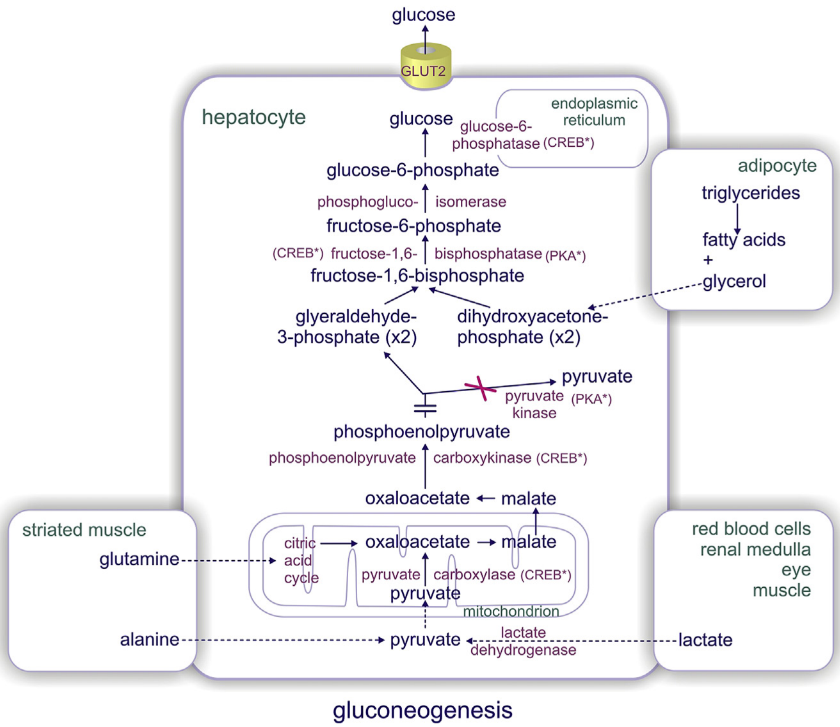


FIGURE 7-2 Metabolic events of gluconeogenesis (initiated when blood glucose levels decline below a threshold). The pathway is fed by different sources: striated muscle, adipocytes, and through lactate from various tissues. The metabolites enter the pathway at different levels. The enzymes whose activity is regulated by protein kinase A are indicated by a star (PKA*). Of these, pyruvate kinase is inhibited, to prevent a cyclic reaction, whereas fructose-1,6-bisphosphatase (FBP) and glucose-6-phosphatase are stimulated. The transcription factor CREB stimulates expression of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK), and FBP (indicated by CREB*).

the islets of Langerhans). It is released when the concentration of glucose in the bloodstream falls roughly below 3 mmol/l (which is 540 mg/l). Red blood cells and the brain are particularly sensitive to glucose levels because the first lack the enzymes required to breakdown fatty acids (β-oxidation) and the second is poorly provided with fatty acids because of limited transport across the blood–brain barrier (double barrier, the plasma membrane of endothelial cells and astrocytes that surround them). In the first instance the arrival of glucagon at the liver causes the conversion of stored glycogen into glucose (glycogenolysis), normally lasting for some 24 h before the liver stock is entirely depleted. Adrenaline brings about the same process in the muscle through the β2-adrenergic receptor (ADRB2) (return to Chapter 2, "An Introduction to Signal Transduction", Figure 2-15) and to some extent in the liver (via the α1-adrenergic receptor, ADRA1). It is important to remember that in contrast to the liver, muscles do not share their glucose with other body parts because they lack expression of glucose-6-phosphatase. They can share in an indirect way, through the production of lactate (anaerobic metabolism assuring the production of ATP without oxidative phosphorylation) which is converted into glucose by the liver and kidney. After a delay, glucagon next sets in train the process of gluconeogenesis, which provides a source of glucose for the entire body. Typically, in overnight-fasted volunteers, net glycogenolysis accounts for 45% of the overall release of glucose into the circulation, whereas 55% is on account of gluconeogenesis. The production peaks at two days of starvation and then lowers gradually (Figure 7-3) (Cahill, 2006; Chandramouli et al., 1997). In the long run, starting at three days of starvation, ketone bodies, β-hydroxybutyrate, and acetoacetate, in part replace glucose in order to moderate muscle wasting (alanine is an important source and this may lead to loss of muscle at a rate of up to 20 g/day). Ketone bodies are produced by the liver from fatty acids and reduce the fat content of adipocytes. Upon arrival in target tissues, they are converted back into acetyl CoA which then enters the citric acid cycle and is oxidized in the mitochondria for the production of ATP. The brain also uses ketone bodies for the production of long-chain fatty acids (for the maintenance of its membranes). Gluconeogenesis is boosted by glucagon-stimulated expression of key enzymes of which we mention pyruvate carboxylase (PC, matrix mitochondrion), phosphoenolpyruvate carboxykinase-1 (PCK1, cytoplasm), fructose-1,6-bisphosphatase (FBP1/2, cytoplasm), and glucose-6-phosphatase (G6PC, transmembrane endoplasmic reticulum). Importantly, prolonged fasting also causes the release of cortisol (glucocorticoid) from the adrenal gland, and it contributes to the increase in expression of gluconeogenic genes with an additional role in the elevation of expression of GLUT2, the (passive) bidirectional glucose transporter of the liver. Moreover, cortisol increases glucagon release from the endocrine pancreas (Wise et al., 1973; Altarejos and Montminy, 2011).

Starvation studies involved graduate students, diabetes type II patients and lasted for slightly over a week. Obese people have also been the subject of these studies and they were kept without food for five to six weeks! Figure 7-3 is largely based on the data obtained from this group. From two weeks onward the brain relies predominantly on β-hydroxybutyrate and acetoacetate because the glucose production is reduced. The renal medulla (low oxygen supply), red blood cells (no mitochondria), cells that constitute the eye lens, and the outer segment of the retina (hardly any mitochondria) rely on glucose and glycolysis for ATP production because they lack the tricarboxylic acid cycle (TCA, Krebs, or citric acid cycle) or the supply of oxygen is too low to use the metabolites of the TCA cycle

(NAD^+ and $FADH^+$) in the electron transport chain. The resulting pyruvate is converted into lactate and reused by the liver for gluconeogenesis. A final point, protein-only diets do not provide sufficient levels of glucose or ketone bodies because of a limited metabolic rate. Fat and sugars are essential components of a balanced diet (El Bacha et al., 2010).

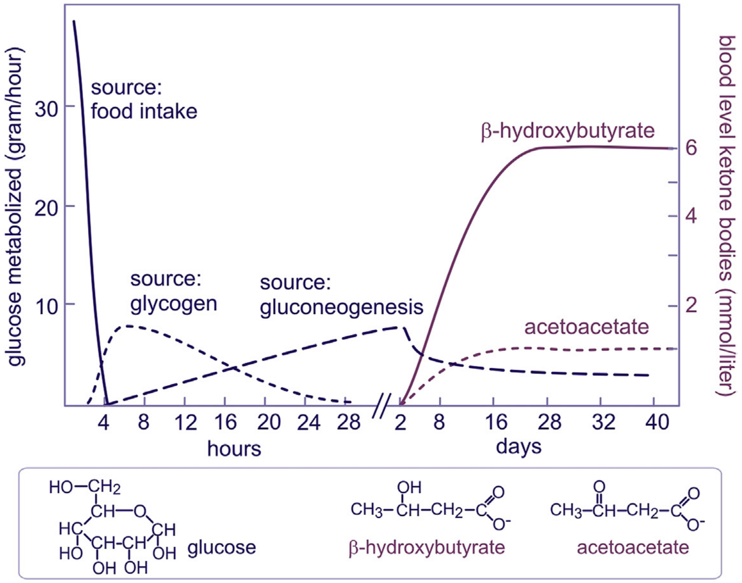


FIGURE 7-3 Metabolic stages at the onset of starvation. Initially glucose is provided through glycogenolysis in the liver (which carries a stock of ~70 g of glycogen). A slow onset gluconeogenesis starts after a couple of hours and peaks at 2 days, with the liver being the main supplier (75%) and the renal cortex taking an honorable second place (25%). Gluconeogenesis attenuates after two days of fasting and this is compensated by the production of "ketone bodies" (acetoacetate and β-hydroxybutyrate). These are mainly used by the brain, fueling about 60% of the required metabolites for ATP production. The remaining 40% continues to be provided by glucose. Other tissues, in particular muscle, will use fatty acid oxidation as the main source for ATP production with the exception of red blood cells, which lack mitochondria. Certain cells have low numbers of mitochondria or reduced oxygen supply and they too continue to rely on glucose. *Image adapted from Cahill (2006).*

SIGNALING THROUGH THE GLUCAGON RECEPTOR

Glucagon is a peptide hormone that folds into a single α-helix. It is cleaved from a small precursor protein named GCG (amino acids 53–81). It binds the extracellular segment of a B-type G-protein-coupled seven transmembrane-spanning receptor, named glucagon receptor, GCGR

(Koth et al., 2012). B-type receptors, also known as secretin-type receptors, have a large binding pocket in the transmembrane helix-bundle and a large extracellular domain that participates in ligand binding (Figure 7-4). The glucagon receptor is coupled to heterotrimeric G-proteins, mainly Gαs (GNAS1) and to a lesser extent to Gαi (GNAI) or Gαq (GNAQ). In the liver, the action of Gαs dominates and binding of glucagon results in activation of adenylyl cyclase. This, in turn, gives rise to an increased level of intracellular cAMP, a second messenger that binds the regulatory subunit (PRKAR1A) of PKA (PRKACA). The holoenzyme is a tetramer, comprising two regulatory and two catalytic subunits (see Figure 7-6(c)). The regulatory subunits wrap around the catalytic subunits and prevent enzyme activity. The binding of cAMP is cooperative, so that occupation of the first site by cAMP enhances the affinity of the vacant site (Doskeland et al., 1993). With both binding sites occupied, the stability of the holoenzyme declines by a factor of 10^4, due to conformational changes in the regulatory subunits, so liberating the protein kinase. It is now able to phosphorylate downstream targets.

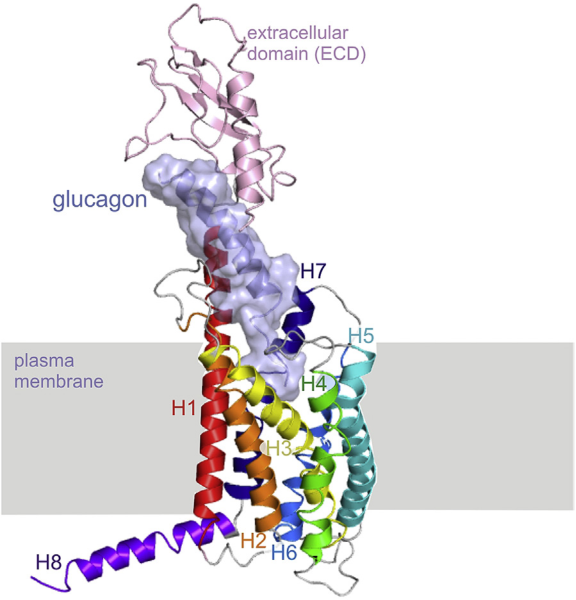


FIGURE 7-4 Predicted structure of the glucagon receptor (GCGR). Note the extended helix H1 (in red) which provides support for the extracellular domain and note the wide binding pocket in the transmembrane helix-bundle, typical of B-type G-protein-coupled receptors. The image represents a theoretical model based on X-ray structures, site-directed mutagenesis data, SAR data, and energy-based conformational modeling. The GCGR-glucagon model (described in Siu et al. (2013), pp. 444–449) was kindly provided by Stevens and coworkers.

PROTEIN KINASE A

PKA generally refers to the catalytic subunit of a tetrameric complex comprising two regulatory and two catalytic subunits (the holoenzyme). The human genome contains three isoforms of PKA (PRKACA (PKAα), PRKACB (PKAβ), and PRKACG (PKAγ)) and four isoforms of the regulatory subunit PKRAR1A (RIα), PKRAR1B (RIβ), PRKAR2A (RIIα), and PPRKAR2B (RIIβ). In the context of gluconeogenesis, we will mainly deal with PKAα and RIα (both expressed in human liver). PKA is a broad spectrum kinase because it recognizes (and phosphorylates) serine or threonine residues embedded in the sequence RRxS/Tx ("x" is variable) and this is rather widespread (Figure 7-5, PRKACA "phosphosite sequence logo"). What distinguishes the different forms of PKA are the tissue-specific regulatory subunits (RI or RII) with which they are associated and the binding of these regulatory subunits to specific anchoring proteins, the so-called A-kinase anchor proteins (AKAPs). These locate the holoenzyme in specific subcellular regions and bring specific substrates in the vicinity (or out of reach) of the enzyme (Scott et al., 2013).

PKA belongs to the subfamily of ACG kinases, named after protein kinase A, -C, and -G, which are grouped together because of overall sequence homology, a characteristic C-terminal (domain referred to as "AGC-kinase C-terminal") and because they share similar mechanisms for rendering the kinase catalytically competent (Kannan et al., 2007). For more information about the protein kinase families, return to Chapter 2 "An Introduction to Signal Transduction," sections "Protein kinases catalyze the phosphate transfer" and "Protein kinase activation mechanisms." Briefly, PKA is composed of two lobes, the N- and C-lobe, which are surrounded by two tails, the C- and N-terminal tails. As mentioned, the C-terminal tail is common to all ACG kinases, but the N-terminal is specific for PKA. We note that both the N-terminal and C-terminal tails carry sequence motifs that interact with other proteins (not necessary substrates) (see Figure 7-5). The catalytic cleft is situated at the interface of the two lobes. The kinase phosphorylates its own C-terminal tail (serine-338) during synthesis (cotranslational) and undergoes a second phosphorylation in the activation segment situated in the catalytic cleft (threonine-197) (posttranslational). The constitutively active 3-phosphoinositide-dependent protein kinase-1 (PDPK1) is generally considered to be the master-switch kinase for members of ACG family of protein kinases (Dettori et al., 2009). These phosphorylation sites are rather stable; enzyme activity is not subject to regulation by protein phosphatases (enzymes that remove the phosphates). The phosphorylated protein kinase is catalytically competent, which means that: it can bind substrate and that ATP is properly oriented so that the γ-phosphate positions near (within 0.3 nm) the OH-group of the serine or threonine of the substrate and near the O^--group of

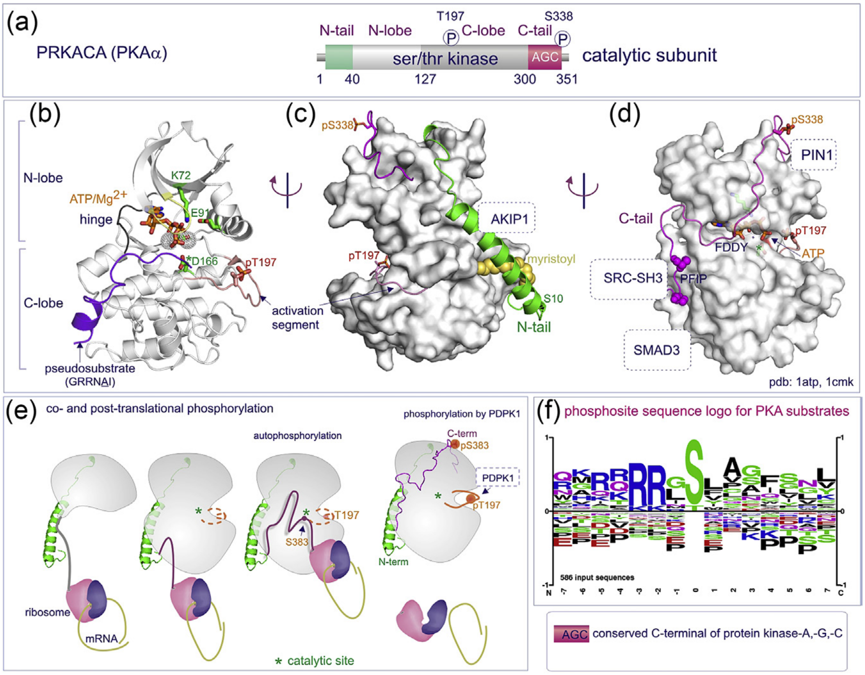


FIGURE 7-5 Molecular characteristics of the catalytic subunit of protein kinase A (PKA). (a) Domain architecture of the catalytic subunit of PKA. (b) PKA displays a typical kinase fold, with an N- and C-lobe connected by a hinge. The N-lobe essentially coordinates the positioning of ATP, whereas the C-lobe carries the catalytic residue and takes care of substrate binding. The enzyme shown here is bound to a short pseudosubstrate (in purple) which carries the sequence RRxAx rather than the consensus phosphosite-sequence RRxS/Tx. Amino acids highly conserved among protein kinases are highlighted by stick representations. Of these, K72 and E91 are essential for the correct positioning of ATP, D166 is the catalytic residue, and phospho-T197 plays a role in correctly positioning of the activation segment and, as a consequence, the positioning of the entire N-lobe relative to the C-lobe ("closed conformation"). (c) The N-terminal sequence (N-tail) wraps around the N- and C-lobe and may contain a myristoyl group which is buried inside the enzyme. The N-tail contains a binding site for the kinase-interacting protein AKIP1 which localizes protein kinase A (PKA) to mitochondria in the heart. (d) The C-terminal sequence (C-tail) also wraps around the C- and N-lobe. Sequence motif FDDY plays a key role in the correct positioning of ATP. The proline-rich motif PFIP binds proteins with an SH3-domain, such as the tyrosine protein kinase SRC. Phosphorylation of S338 in the C-tail is essential for the correct conformation of the N-lobe, in particular, with respect to bringing about the right orientation of K72 and E91. (e) PKA undergoes two phosphotransfers, both are necessary for rendering the enzyme catalytically competent. One occurs in the C-terminal tail during translation (cotranslational), and is most likely the consequence of autophosphorylation, and the second occurs in the activation segment after protein translation (posttranslational). The second phosphotransfer is brought about by the 3-inositol-dependent protein kinase D1 (PDPK1). Ribosome and PKA are not drawn to scale. (f) "Phosphosite Sequence logo" of PKA substrates as revealed by mass spectrometric analysis of 586 input sequences. Positive values indicate the prevalent detection of amino acids within the phosphosite (you nearly always find an R(arg) two amino acids upstream of the phosphoserine or -threonine), whereas negative values indicate their absence (you would never find an E(glu) one amino acid upstream). The consensus sequence for PKA is xRRxS/Tx but, as the sequence logo shows some variation exists. *The image is obtained from the PhosphoSitePlus database (Hornbeck et al., 2012).*

the catalytic aspartate. It remains active until the catalytic subunit encounters a regulatory subunit with which it forms a complex (Figure 7-6). The holoenzyme is inactive and below we will discuss why.

Regulatory subunits are characterized by a dimerization domain (D/D), an intrinsically disordered linker region which contains a consensus (pseudo)phosphosite and two cyclic-nucleotide-binding domains (CNA and CNB). Sequence divergence between the four isoforms mainly occurs in the linker region. For type-I regulatory subunits, the phosphosite is a pseudosubstrate, the serine being replaced by an alanine or a glycine, for type-II regulatory subunits it is a genuine phosphosite (Figure 7-6(a)). The cyclic-nucleotide domains are connected by a flexible helix. The relative position of these two domains is determined by cAMP. The helix is kinked in the presence of cAMP and as a consequence the two nucleotide-binding domains show extensive contacts. The helix straightens in the absence of cAMP, separating the two domains. The intrinsically disordered linker interacts with the catalytic cleft of the catalytic subunit in the absence of cAMP, and the ensuing structural reorganization helps to wrap the nucleotide-binding domains around the C-lobe of the catalytic residue (Akimoto et al., 2013). The complex only dissociates in the presence of cAMP.

For the type-I regulatory subunits, binding of the pseudosubstrate by itself explains catalytic inhibition but it fails to explain inhibition by type-II regulatory subunits. Therefore, the extensive contacts between the regulatory and catalytic subunit must also bring about conformational changes (allosteric changes) that either interfere with kinase activity or prevent dissociation of the regulatory subunit after its phosphorylation. Such allosteric changes were made evident for PKA associated with RIIα, where it was shown that the regulatory subunit holds the catalytic domain in an open, catalytically noncompetent state, thus preventing phosphorylation of the phosphosite (Wu et al., 2007; Taylor et al., 2012). RIIβ acts in a different fashion because the kinase occurs in a closed state, meaning catalytically competent, and indeed the regulatory subunit is effectively phosphorylated. Normally phosphorylation should destabilize the interaction with the catalytic subunit, otherwise “real” substrates would remain attached for a very long time, but in the case of RIIα this does not occur spontaneously. Separation still requires the presence of cAMP. Whether or not RIIβ has to be repeatedly dephosphorylated in order to fulfill its regulatory role remains unclear. Besides the differences in regulatory mechanisms, structural analysis has also revealed that different regulatory subunits bring about different assemblies (quaternary structures) of the holoenzyme (Taylor et al., 2012). Moreover, holoenzymes display different sensitivities for cAMP and gene knockout studies have revealed that they are functionally nonredundant.

Importantly, the regulatory subunits attach to A-kinase anchor proteins which sequester the holoenzyme to specific subcellular locations.

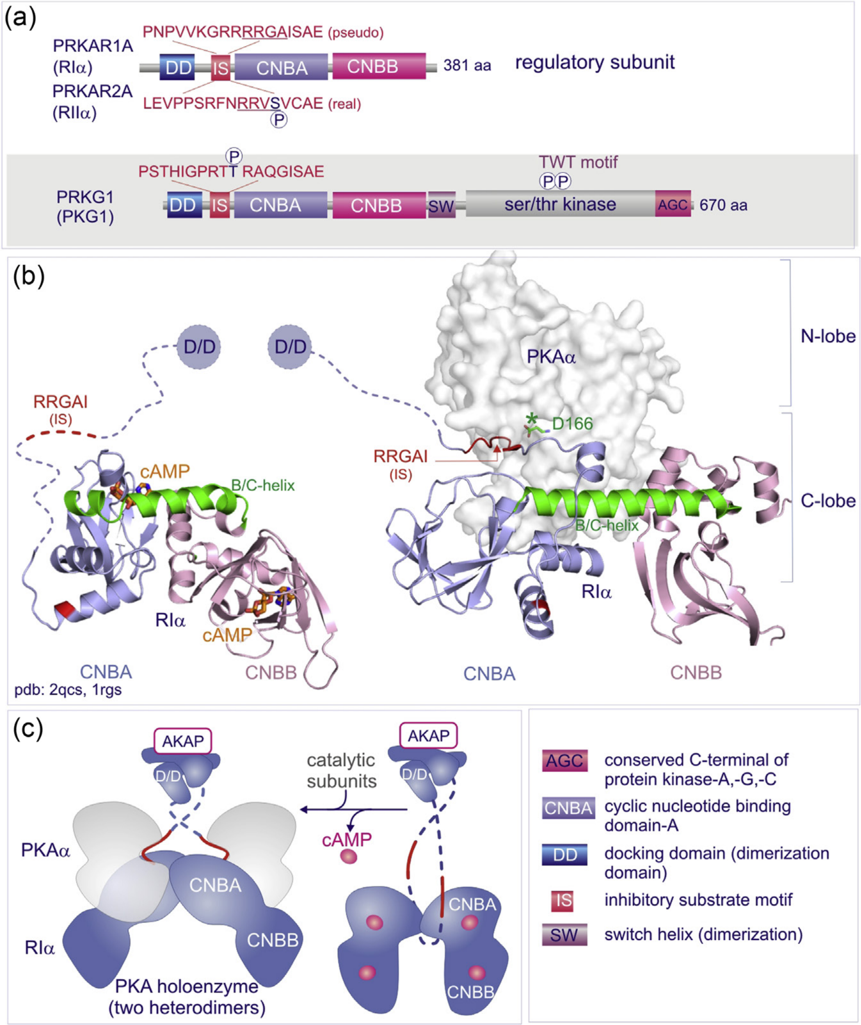


FIGURE 7-6 Structure of the protein kinase A (PKA) regulatory subunit and assembly of the holoenzyme. (a) Domain architecture of the regulatory subunit of PKA (PRKAR1A and -2A). For comparison, the domain architecture of protein kinase G (PRKG1) is shown underneath. Note that for the cyclic-GMP-dependent protein kinase, the regulatory- and catalytic-subunits are combined in one gene. The amino acid sequences in red are the (pseudo) phosphosites (IS) in the linker region. (b) The regulatory subunit RIα can adopt two conformations: on the left in the presence and on the right in the absence of cAMP and being attached to a catalytic subunit. Note how the cyclic nucleotide-binding domains make extensive contacts with each other in the presence of cAMP and how, when cAMP is removed, the B/C helix (in green) separates the two domains and allows them to interact with the C-lobe of the catalytic subunit. The pseudo-phosphosite (RRGAI) positions in the catalytic cleft right in front of the catalytic aspartate residue (D166). Only one of the two regulatory subunits is shown. (c) Schematic representation of the PKA holoenzyme, comprising a tetramer of two catalytic and two regulatory subunits. The regulatory subunits remain attached to each other in the presence of cAMP. Note the binding of AKAP to the dimerization domain (D/D). It directly or indirectly localizes the PKA holoenzyme to a specific subcellular localization (see also Figure 7-7).

The hallmark motif in AKAP is an amphipathic helix that docks with high affinity to the dimerization domain (D/D) of the regulatory subunit. Some 29 AKAPs are recognized by the HUGO gene nomenclature committee, but which of these play a role in gluconeogenesis is not clear.

PROTEIN ISOFORMS

A number of proteins involved in signaling pathways exist in different isoforms, produced by very closely related gene duplicates. It is not always clear from the literature which of these isoforms is concerned. Information can be obtained from proteomics databases but this is not necessarily satisfactory for two reasons. First, signaling events are organized in a spatial manner and low-abundant isoforms may play a key role because they are coupled to the ion channel, the receptor, or the specific subcellular compartment in question (via adaptor proteins) and second, the relative abundance of proteins may vary under different conditions and in different cell types that make up the tissue (like the liver). Moreover, functional models may be constructed on the basis of a heterogeneous set of data, derived from studies with mice, rats, and humans. With respect to the human liver, from the three isoforms of the cAMP-dependent protein kinase, PRKACA (PKAα) is most abundantly expressed, whereas PRKACB (PKAβ) is only weakly present and PRKACG (PKAγ) seems absent. Of the four regulatory subunits, PRKAR2A (RIIα) is most abundant, followed by PRKAR1A (RIα), and the others are hardly detectable (information from PaxDB; see below). Incongruences may occur between findings in articles and information in annotated databases. For example, whereas the literature mentions the involvement of CRTC2 as the coactivator of CREB in the process of gluconeogenesis (Altarejos and Montminy, 2011), proteomic data reveal a lack of CRTC2 expression in the human liver but a moderate presence of CRCT1. When proteins are indicated in a "generic fashion," such as CREB, PRKAC (PKA), CRTC, etc., it means that we are not able to specify the isoform. We note that besides isoforms, numerous alternative sequences exist due to alternative splicing. Thus, PRKACB has 10 variants (PRKACB1–10), which mainly differ in their N-terminal sequence.

Web resource

PaxDB is a comprehensive absolute protein abundance database, which contains whole genome protein abundance information across organisms and tissues (Wang et al., 2012a): http://pax-db.org/#!home

AKAP, ANCHORING AND SCAFFOLDING

To illustrate how AKAP takes a central role in the assembly of macromolecular signaling complexes and how these may be instrumental in very precise control of the subcellular localization and conductive properties of the AMPA receptor (glutamate-gated ion channel), we provide the example of AKAP79 in neuronal synapses. For more information about the AMPA receptor, return to Chapter 4 "Cholinergic Signaling and Muscle Contraction," section "Neurotransmitters that function with both ionotropic and metabotropic signaling mechanism," Figure 4-28. The AMPA receptor is a glutamate-gated anion channel (Na^+, K^+, or Ca^{2+}) composed of four subunits (giving rise to a heterotetramer). Heterotetramers, which comprise subunit-2, often present in the synaptic cleft, are impermeable to Ca^{2+}.

It plays a role in the plasticity of synaptic transmission. In the process of long-term potentiation, AMPA receptors translocate from nonsynaptic to synaptic sites, providing a cell biological basis for controlling the synaptic level of AMPA receptors and hence postsynaptic responsiveness (Lüscher et al., 2000). It is not clear whether the regulated "synaptic delivery" of AMPA receptors is mediated by exocytosis from intracellular pools directly into the postsynaptic membrane, or by lateral translocation from extrasynaptic regions on the neuronal surface, or both. Channel conductivity and its recruitment are controlled by the phosphorylation state of the receptor (phosphorylation sites in C-terminal region). Two protein kinases play a role in the translocation into the postsynaptic membrane, PKA, and type-II Ca^{2+}-calmodulin-dependent protein kinase (CAMKII) (Esteban et al., 2003).

AKAP79 is attached to the PKA holoenzyme, via RIIβ, and to calcineurin, a multisubunit Ca^{2+}/calmodulin-sensitive serine/threonine protein phosphatase (PPP3CA) (Figure 7-7). It is also attached to Disc Large homolog-1 (DLG1, also known as SAP97) which basically is an adaptor protein that carries a number of protein–protein interaction domains. One of these domains, named PDZ, attaches to the C-terminal sequence (-ATGL) of the AMPA receptor (subunit GRIA1) and thus connects the signaling complex with the receptor (Colledge et al., 2000). Both the protein kinase and the phosphatase now compete for access to a neighboring (phospho)serine (S683). AKAP79 also attaches to a Ca^{2+}-sensitive adenylyl cyclase (ADCY8) and a highly localized production of cAMP occurs in the synapse at sites where Ca^{2+} enters, that is, at the NMDA receptor (Willoughby et al., 2010). A similar setup has been discerned in heart myocytes where evidence has been provided that AKAP79 connects a voltage-gated Ca^{2+} channel (CACNA1C) together with calcineurin and the PKA holoenzyme (Zhang et al., 2012; Oliveria et al., 2007). In short, macromolecular signaling complexes are assembled through anchoring/scaffolding proteins. These complexes comprise both the sources of production of second messengers (or in the case of Ca^{2+} the presence of an ion channel) as well as the corresponding effectors (cyclases, kinases, and phosphatases) and substrates (receptors, ion channels, or enzymes).

For more information about AKAP-signaling complexes and synaptic plasticity, we refer to Sanderslon and Dell'Acqua (2011).

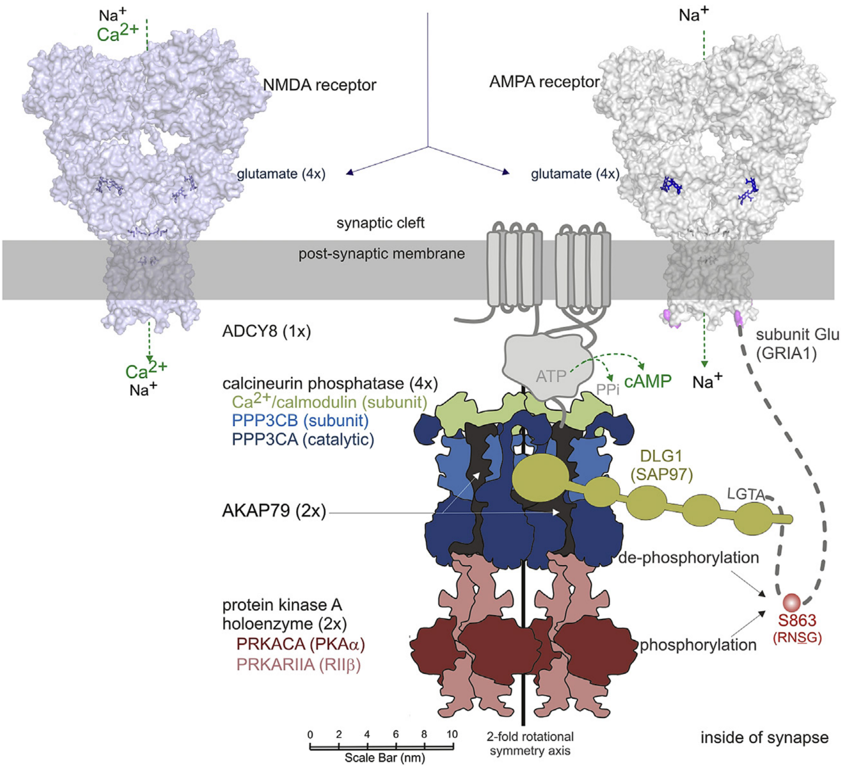


FIGURE 7-7 AKAP anchoring and scaffolding function in the postsynaptic membrane. Two copies of the A-kinase anchor protein-79 (AKAP79) take central stage in the assembly of a large macromolecular signaling complex, consisting of two PKA holoenzymes, four calcineurin phosphatases as well as the adaptor protein DLG1 which, in turn, is attached to the ionotropic glutamate receptor (AMPA receptor or GRIA). AKAP79 has also been reported to attach to the Ca^{2+}-sensitive adenylyl cyclase-8 (ADCY8) thus ensuring a highly localized production of cAMP. The complex brings the protein kinase A and the phosphatase calcineurin in the vicinity of a crucial serine residue (ser-863) of the AMPA receptor and this must allow for a very rapid and precise control of the phosphorylation state of the receptor. The phosphorylated receptor shows enhanced conductivity for anions and translocates into the synaptic cleft. Two important second messengers, Ca^{2+} and cAMP are shown in green. The graphic representation of the AKAP macromolecular complex is kindly provided by Matthew Gold, Department of Neuroscience, Physiology and Pharmacology, UCL, London, UK (Gold, 2012).

ACTIVATION OF PKA BY cAMP

PKA is activated through cAMP-mediated dissociation from the regulatory subunit (Taylor et al., 2012). The catalytic subunit remains active as long as cAMP prevents its recapture. What this means is that short pulses of cAMP would only allow phosphorylation of nearby substrates, whereas long pulses of cAMP may give rise to a much broader substrate range. The duration and concentration of the cAMP pulse is determined by its production by adenylyl cyclase (ADCY) and its removal by cAMP phosphodiesterase (PDE). Diffusion of PKA into the cell will also depend on the number of possible substrates in its immediate environment. Frequent encounters followed by phosphate transfer must reduce the diffusion rate (it is like speaking to lots of people on your way out of a meeting, the more people you meet the longer it takes to get out of the room). PKA is unique among the protein kinases in that the catalytic and regulatory entities are separated. For near all other protein kinases, they are part of the same protein. We have seen the example of the cGMP-dependent protein kinase (PKG or PRKG) and of the tyrosine protein kinase SRC (return to Chapter 4, “Cholinergic Signaling and Muscle Contraction,” Section “The cGMP pathway leading to smooth muscle relaxation” and Figure 4-25 and return to Chapter 2, “An Introduction to Signal Transduction,” Section “Posttranslational modifications involved in signaling events” and Figure 2-21). Another example is protein kinase C, member of the ACG-protein kinase family, which carries a regulatory pseudosubstrate in its N-terminal sequence (discussed in Chapter 9, “Protein Kinase C and Cell Polarity”).

Web resources

- HUGO gene nomenclature committee database of A-kinase anchor proteins: http://www.genenames.org/genefamilies/AKAP
- Protein kinase resource from Purdue University, the San Diego Supercomputer Center and the University of California at San Diego: http://pkr.genomics.purdue.edu/pkr/Welcome.do

REFRESH YOUR UNDERSTANDING

For PKA and its regulation, return to Chapter 2 “an introduction to signal transduction,” Figures 2-35 and 2-4.

For the role of AKAPs in glycogenolysis, return to Chapter 2 “an introduction to signal transduction,” Figure 2-42.

REFRESH YOUR UNDERSTANDING *(cont'd)*

For GPCR receptors, return to Chapter 5 "Sensory Signal Processing," Figures 5-20 to 5-23.

For adenylyl cyclases, return to Chapter 3 "Regulation of Muscle Contraction by Adrenoceptors," Figures 3-11 to 3-14.

For phospholipases, return to Chapter 3 "Regulation of Muscle Contraction by Adrenoceptors," Figures 3-15 and 3-16.

For phosphodiesterases, return to Chapter 4 "Signaling pathways operated by acetylcholine," Figures 4-15 to 4-18.

For the process of glycogenolysis, return to Chapter 2 "An Introduction to Signal Transduction," Figures 2-16 and 2-17.

PKA SUBSTRATES INVOLVED IN GLUCONEOGENESIS

Among the targets of PKA is an enzyme involved in gluconeogenesis namely FBP2 and phosphorylation increases its activity. As a consequence, more glucose can diffuse out of the liver (return to Figure 7-2). Pyruvate kinase is also phosphorylated and this causes its inactivation (exclusively in the liver). As a consequence, phosphoenolpyruvate is not converted into pyruvate (last step in the glycolysis pathway) and instead enters the gluconeogenesis pathway. PKA also phosphorylates and stimulates glycogen phosphorylase kinase which results in activation of glycogen phosphorylase and subsequent activation of glycogenolysis (removing glucose from glycogen). Glycogen synthase is phosphorylated and inhibited by PKA and this blocks glycogen synthesis. In order to prevent dephosphorylation by serine/threonine phosphatases, the inhibitory subunit I-2 (PPP1R1A) is phosphorylated and this causes sequestration of the catalytic subunit PPP1CA. As a last cytoplasmic target, we mention the protein kinase SIK2, it is inhibited through phosphorylated on serine-587 within a conserved RRA$\underline{\text{S}}$D motif.

Web resource

- PhosphoSitePlus, a protein modification resource, allows you to search for PKA substrates (type search term "PKACA"). As you will learn, the number of substrates is very large indeed: http://www.phosphosite.org/homeAction.do

CREB, A NUCLEAR TARGET OF PKA

With sufficient intracellular cAMP, the free catalytic subunit is able to translocate to the nucleus, without being recaptured by regulatory subunits, there to find an important target, the protein CREB1 (Harootunian et al., 1993; Hagiwara et al., 1993). Alternatively, with increasing concentrations of cAMP, some of the second messenger diffuses into the nucleus there to activate nuclear-resident PKA (Sample et al., 2012) (Figure 7-8). CREB1 is associated, in the form of a homodimer, with DNA in gene enhancer regions that contain the cAMP response element (CRE) that either appears as a palindromic DNA sequence TGACTGAC/CAGTCAGT (example from the somatostatin gene) or half-site sequence (TGACG or CGTCA) (Montminy et al., 1986; Comb et al., 1986). Phosphorylation of CREB1 on serine-133, in the sequence -RRPS̲Y- by PKA creates a new interaction surface which causes the binding of the CREB-binding protein (CREBBP) or of EP300 (homologous protein). Both are essential coactivators in the ensuing process of enhanced gene transcription (Parker et al., 1996). As a consequence of this, expression increases for a number of enzymes that play a role in gluconeogenesis, among these are PC, PCK-1, FBP, and glucose-6-phosphatase. Phosphorylation of CREB also enhances expression of the coactivator PGC1α (PPARGC1A) and the nuclear receptor NR4A1 (orphan receptor). These too increase expression of a number of genes and collectively they raise gluconeogenic activity thus allowing for a sustained production of glucose by the liver.

THE PRESENCE OF A SUITABLE RESPONSE ELEMENT IS NOT A GUARANTEE FOR CREB-MEDIATED REGULATION OF GENE EXPRESSION

Most of the 750,000 palindromic and half-site CREs of the human genome are unoccupied in cells owing to disruptive cytosine methylation of the CREB-binding site (Iguchi-Ariga and Schnaffer, 1989) (these occur in silenced or near-silenced genes). Unmethylated functional CREB-binding sites occur in about 5000 genes or roughly one-quarter of the mammalian genome (Impey et al., 2004). CREB-binding sites are most effective when placed near the TATA box, within a distance of 250 base pairs, and they become progressively weaker when moved further upstream (De Cesare et al., 1999). CREB binds to a majority of the 5000 sites and undergoes Ser133 phosphorylation in response to elevated levels of cAMP. Despite extensive CREB phosphorylation, only about 100 target genes display increased gene transcription and the pattern of genes concerned differs considerably between cell types (Impey et al., 2004). The restricted number of phospho-CREB-activated genes may be explained in part by the lack of a TATA box, something that occurs in

THE PRESENCE OF A SUITABLE RESPONSE ELEMENT IS NOT A GUARANTEE FOR CREB-MEDIATED REGULATION OF GENE EXPRESSION *(cont'd)*

9 out of 10 genes, but other mechanisms must play a role as well in order to explain the variation between different cell types (cooperation with other transcription factors (among which specific pioneer-transcription factors) or the absence or presence of specific repressors or activators).

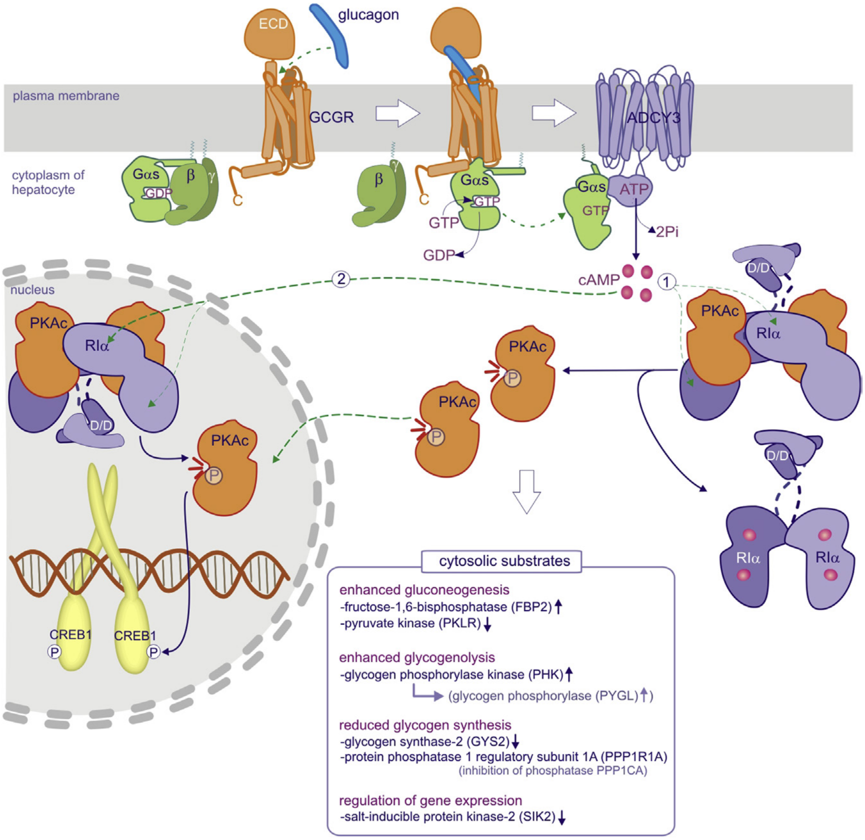

ECD
glucagon
plasma membrane
GCGR
ADCY3
cytoplasm of hepatocyte
Gαs
GDP
β
γ
C
GTP
ATP
2Pi
GDP
cAMP
①
②
nucleus
PKAc
RIα
D/D
P
CREB1
cytosolic substrates
enhanced gluconeogenesis
-fructose-1,6-bisphosphatase (FBP2)↑
-pyruvate kinase (PKLR)↓
enhanced glycogenolysis
-glycogen phosphorylase kinase (PHK)↑
(glycogen phosphorylase (PYGL)↑)
reduced glycogen synthesis
-glycogen synthase-2 (GYS2)↓
-protein phosphatase 1 regulatory subunit 1A (PPP1R1A)
(inhibition of phosphatase PPP1CA)
regulation of gene expression
-salt-inducible protein kinase-2 (SIK2)↓

FIGURE 7-8 Signaling pathway of the glucagon receptor. Glucagon binds its receptor, a B-type G-protein-coupled receptor. The ensuing conformational changes at the cytosolic side create a binding site for the Gαs subunit of a heterotrimeric G-protein. GDP is subsequently exchanged for GTP and, as a consequence, Gαs dissociates from its Gβγ partners and instead binds to and stimulates adenylyl cylase-3 (ADCY3). This results in the production of the second messenger cAMP. Binding of cAMP to the regulatory subunits of PKA (RIα) leads to the dissociation of the catalytic subunit (indicated as PKAc). The now active protein kinase phosphorylates a number of enzymes involved in gluconeogenesis, glycogenolysis, and glycogenesis. Its nuclear substrate is the transcription factor CREB. Currently, two models are proposed for the nuclear action of PKA: in the first, the catalytic subunit of PKA migrates into the nucleus (Altarejos and Montminy, 2011) and in the second, cAMP diffuses into the nucleus and activates a nuclear-resident protein kinase-A holoenzyme (Sample et al., 2012). In both instances, the response is substantially delayed, in the order of several minutes, due to slow diffusion.

CREB IS MEMBER OF THE BASIC LEUCINE ZIPPER (bZIP) FAMILY OF PROTEINS

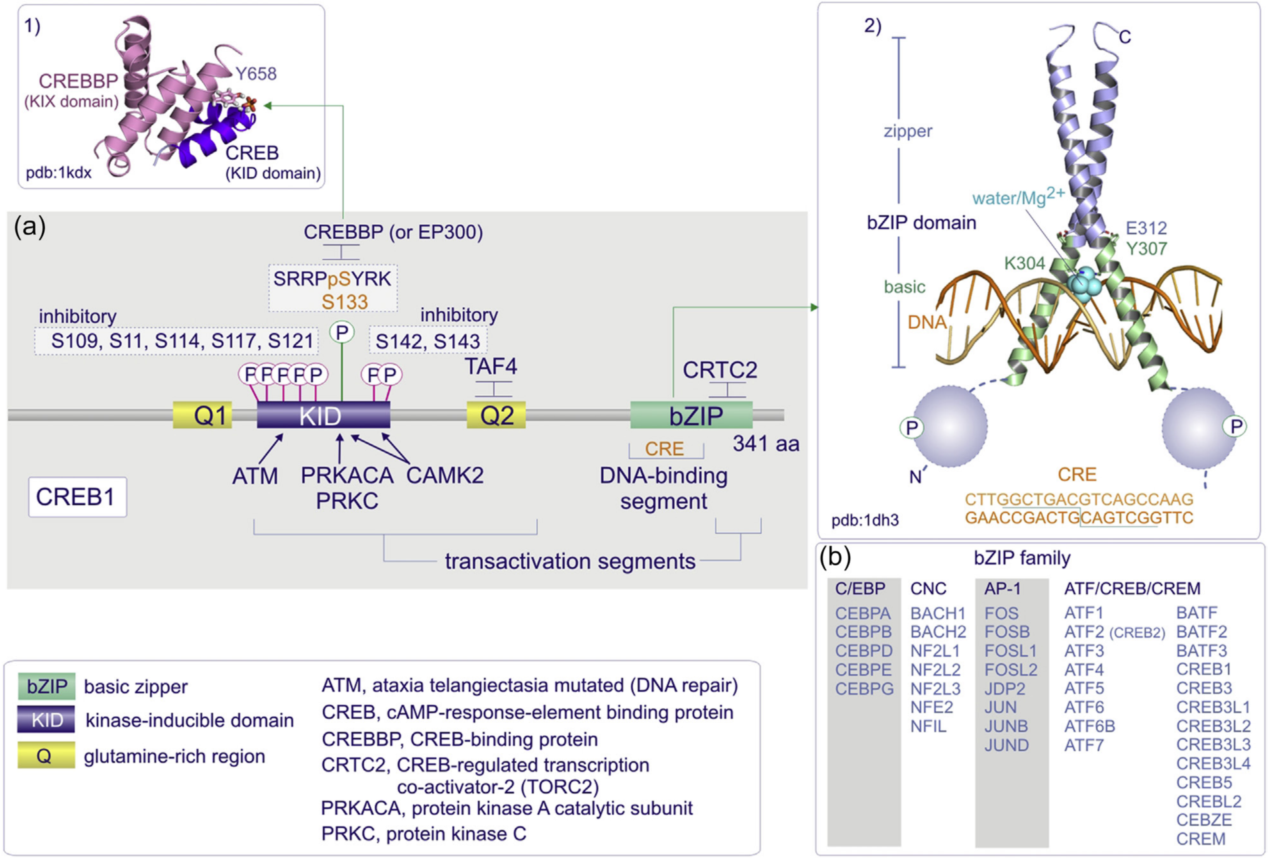
CREB was first described in 1987 as a "cyclic-AMP responsive element" regulating expression of the somatostatin gene (Montminy and Bilezikjian, 1987). It belongs to a larger family of proteins that are grouped together because of a characteristic coiled-coil dimerization motif that is rich in leucines and because of a DNA-binding region rich in basic amino acids, arginine, and lysine, and is therefore named basic leucine-zipper (bZIP). Based on dimer composition and DNA-binding preferences, the family is divided into four subfamilies CNC, C/EBP, AP-1, and CREB/CREM/ATF-1. Homology between the members of the latter subfamily was highlighted by the finding that they were able to functionally compensate each other in mice (knockouts do not show apparent developmental impairments) (Hummler et al., 1994). The zipper region of CREB is six amino acids shorter than that of other members of the family (AP-1, etc.) which makes it energetically unfavorable to form heterodimers. Critical to selective dimerization of CREB/CREM/ATF-1 members are intersubunit hydrogen bonds between the conserved residues tyrosine-307 and glutamate-312 which occur at the interface of the basic- and the zipper-region (Schumacher et al., 2000). Protein dimerization and DNA binding also involves water-mediated interactions (electrostatic and hydrogen bonds) which are brought into place through a hexahydrated Mg^{2+} ion located in the cavity between the bifurcating basic regions and the DNA (Figure 7-9). In the example of the somatostatin cAMP response element (CRE), the Mg^{2+}-mediated interactions enhance DNA binding by more than 25-fold.

KID DOMAIN

The CREB kinase-inducible activation domain (KID, 60 residues) is partially disordered in solution, even when phosphorylated, and undergoes an alpha-helical folding transition upon binding to CREB-binding protein (Radhakrishan et al., 1997).

Web resource

- HUGO gene nomenclature committee, members of the bZIP family: http://www.genenames.org/genefamilies/BZIP



(b) bZIP family

C/EBP	CNC	AP-1	ATF/CREB/CREM	
CEBPA	BACH1	FOS	ATF1	BATF
CEBPB	BACH2	FOSB	ATF2 (CREB2)	BATF2
CEBPD	NF2L1	FOSL1	ATF3	BATF3
CEBPE	NF2L2	FOSL2	ATF4	CREB1
CEBPG	NF2L3	JDP2	ATF5	CREB3
	NFE2	JUN	ATF6	CREB3L1
	NFIL	JUNB	ATF6B	CREB3L2
		JUND	ATF7	CREB3L3
				CREB3L4
				CREB5
				CREBL2
				CEBZE
				CREM

FIGURE 7–9 Con'd

FIGURE 7-9 CREB domain architecture and composition of the bZIP family of transcription factors. (a) Domain architecture of CREB1. The KID domain carries a number of phosphorylation sites for four different protein kinases. Only phosphorylation of serine-133 facilitates binding with CREB-binding protein (CREBBP) or EP300, all other phosphorylation sites are inhibitory. The second glutamine-rich motif (Q2) binds to TAF4, a component of the basal transcription factor complex TFIID. CREB1 contains a DNA-binding segment and two transactivation segments (those that interact with other proteins such as CRTC2, TAF4, and CREBBP). Inset (1) shows the structure of the CREB-KID bound to the CREBBP-KIX domain. The essential interaction between phosphoser-133 and tyr-568 is highlighted. Inset (2) depicts the structure of the bZIP domain, comprising a zipper region, with essential interprotein hydrogen bonds between tyr-307 and E312. Mg^{2+} is coordinated by two lysines (position K304 in each protein) and surrounded by a shell of six water molecules that play a role in dimerization and DNA binding. The N-terminal sequence is indicated by a hypothetical sphere, it contains the phosphorylation sites (KID domain). (b) Members of the bZIP subfamilies of transcription factors (information from Hugo Gene Nomenclature Committee). Their mode of activation does not necessarily involve the same protein kinases and they do not necessarily bind the same response element or interact with the same coactivators (or corepressors).

TRANSCRIPTION AND TRANSCRIPTION FACTORS

The key concepts of regulation of gene transcription stem from the pioneering studies of Francois Jacob and Jacques Monod (Institute Pasteur, Paris, France) in bacterial systems where they showed that genetic control of protein synthesis requires structural genes, which determine the molecular organization of proteins, and regulator and operator genes which control the rate of structural gene transcription through intermediates of cytoplasmic components (repressors). The ensemble of promoter (site where the ARN polymerase binds to DNA), operator, and three structural genes that control lactose metabolism was named the operon ("lac operon") (Figure 7-10(a)) (Jacob et al., 1960). The repressor can be relieved from its function, onset of transcription, or activated, transcription stop (Jacob and Monod, 1961). The model of the lac operon has molded the understanding of gene regulation and opened up the field of gene transcription. The general principle as discovered in bacteria applies to metazoans but with a striking higher level of complexity. Whereas in the majority of cases prokaryotes rely on promoter proximal, topologically restricted cis-elements that drive initiation of transcription, the human genome exhibits multiple composite enhancer elements that situate both at proximal and distal DNA sequences. For example, the gene encoding for Sonic Hedgehog (Shh) in mice is regulated by a distal enhancer that maps nearly one million bases from the promoter (Amano et al., 2009). It is estimated that on average a typical human gene might be regulated by tens of enhancer regions (Bernstein et al., 2012). Moreover, whereas prokaryotes possess readily accessible DNA, in humans (and in fact in all metazoans) the template is wrapped up in nucleosomes which affect the ability of transcription regulators to access their binding sites (Wang et al., 2011; Levine et al., 2014).

The human genome carries ~1200 genes that encode transcription factors. A number of these are lineage-specific transcription factors. They are produced in relatively large quantities, more than 30,000 copies per cell, and through occupation of certain enhancer regions determine the overall transcriptome of the cell in question. In embryonic stem cells, these transcription factors are called "master transcription factors" and examples are OCT4, NANOG, MYOD, C/EPBβ, GATA4, HNF4α, or BRN2. Ectopic expression of master transcription factors causes reprogramming of cell states and can even reprogram differentiated cells into pluripotent stem cells (induced pluripotent stem cells) (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Theunissen and Jaenisch, 2014). Master transcription factors may recruit coactivators, further discussed below, or corepressors such as the polycomb-repressive complexes which silence gene expression by controlling methylation of histone-3 (see Figure 7-11) and ubiquitylation of histone-2 (Holmberg and Perlmann, 2012). The majority of transcription factors are signal-dependent transcription factors and their role is to modulate expression of genes earmarked by the master transcription factors. They occur in lower quantities, in the order of 10,000

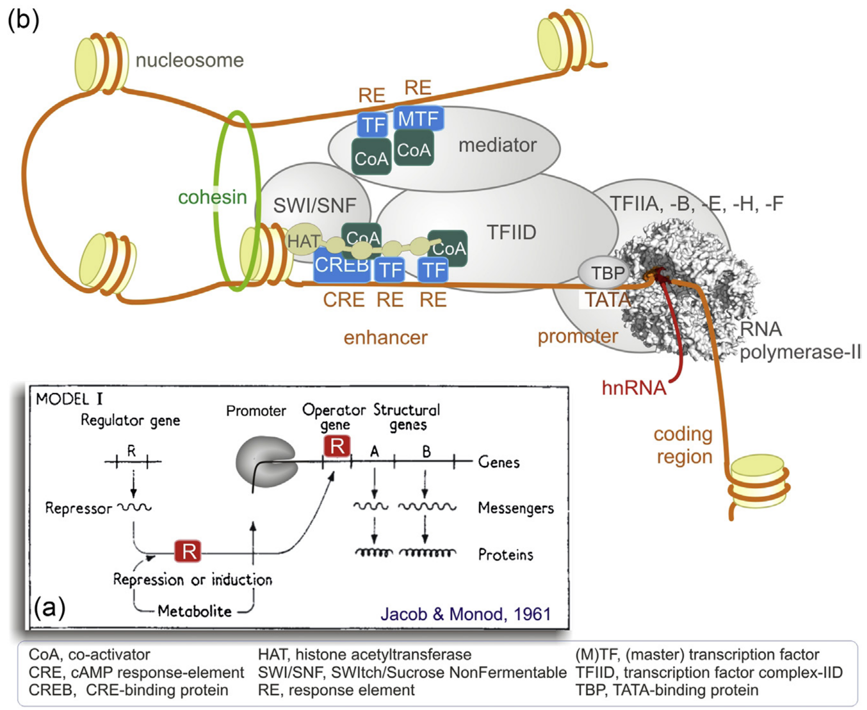


FIGURE 7-10 Regulation of gene transcription. (a) Transcription regulation model proposed by Jacob and Monod for the regulation of lactose-fermenting genes in prokaryotes. The operator is the equivalent of the enhancer. *(Image adapted from the original publication (Jacob and Monod, 1961.)* (b) Transcription factor complexes instrumental in the regulation of gene expression in metazoans. The general distinction is made between regulatory transcription factors (in blue), coactivators (CoA, HAT, SWI/SNF, mediator), and core-promoter factors (TFIID, TFIIA, etc.), all of which play a role in positioning the RNA polymerase II and in initiating the transcription process. Cohesin enhances the efficiency of transcription by stabilizing the DNA loop. Collectively, these components constitute the transcription preinitiation complex (PIC). Another set of transcription factors, the super elongation complex, intervenes in the process of polymerase pause release (not shown).

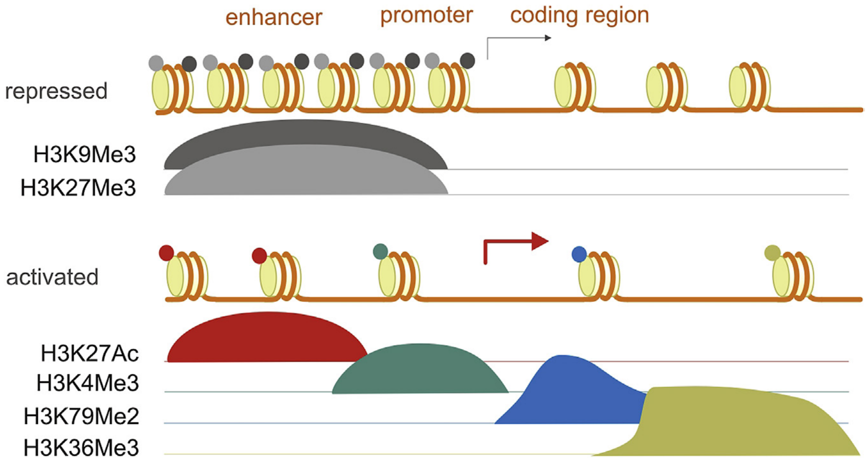


FIGURE 7-11 Acetylation and methylation state of histones in transcriptionally repressed and active genes. *Information from* Levine et al. (2014) *and* Lee and Young (2013b).

copies per cell or less, and their interactivity and/or nuclear localization is controlled by receptor-mediated signaling events (Zhang and Glass, 2013). Members of the CREB family of transcription factors belong to this type.

A NOBEL PRIZE FOR DISCOVERING GENE REGULATION

The Nobel Prize in Physiology or Medicine 1965 was awarded jointly to Francois Jacob, André Lwoff, and Jacques Monod "for their discoveries concerning genetic control of enzyme and virus synthesis."

A NOBEL PRIZE FOR REPROGRAMMING THE TRANSCRIPTOME

The Nobel Prize in Physiology or Medicine 2012 was awarded jointly to John Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent." John Gurdon discovered in 1962 that, under specific conditions, the nucleus of a *Xenopus laevis* oocyte could be replaced with the nucleus from an intestinal cell and provided a first hint that the transcriptome of a differentiated cell can be reprogrammed by components present in the cytoplasm of the oocyte. Shinya Yamanaka discovered 44 years later, in 2006, how differentiated fibroblasts in mice could be reprogrammed to become stem cells by injecting a cocktail of "master transcription factors" such as OCT4, SOX2, KLF4, and MYC.

Transcription factors typically regulate gene expression by binding enhancer elements and recruiting cofactors and RNA polymerase II to the core-promoter elements of genes. Multiple transcription factors typically bind in a cooperative fashion to enhancer elements and regulate transcription of nearby or distant genes through physical contacts that involve looping of the DNA between enhancers and the core-promoter elements. Spatial organization of the DNA (genome topology) plays an essential role in the regulation of gene transcription (Cavalli and Misteli, 2013). As a consequence, most genes are embedded in a milieu of a multitude of enhancers. The core-promoter elements, which include the site of initiation of gene transcription, bind DNA-binding proteins which are referred to as "core-promoter factors" (previously referred to as general transcription factors) (Figure 7-10). We highlight the role of TFIID because it is a principal component of the core complex, responsible for recognizing and binding specific promoter elements. TFIID consists of the TATA-binding protein (TBP) and some 14 conserved TBP-associated factors (TAFs).

Among these is TAF4, a protein that interacts with CREB1. Once TFDII alights on promoter elements, guided by the presence of regulatory transcription factors, it initiates a sequential recruitment of other core-promoter factors including TFIIA, -B, -E, -H, -F, the mediator/CRSP complex and RNA polymerase II. The precise composition of core-promoter factors may differ between cell types and between differentiation states of the same cell type and may even differ between genes. For instance, not all genes carry a TATA box, and promoter regions have other sequences that interact with the core transcription factors and differentially recruit core factors. Regulatory transcription factors, core-promoter factors, and RNA polymerase make up the pre-initiation complex (PIC). Transcription is controlled at two levels: initiation and elongation. Normally, the RNA polymerase II transcribes a short distance and then pauses. The paused polymerase may then transit into active elongation, through a process referred to as pause release, or terminate transcription with the release of a small RNA species. Pause release requires the recruitment of yet another set of proteins, the super elongation complex, which bring about phosphorylation of the polymerase on its long C-terminal tail (which allows the enzyme to escape from the initiation complex and engage in elongation) and of associated "pause factors." For certain genes, in particular those employed during development, regulation occurs at the level of elongation, not initiation. The paused RNA polymerase II keeps the gene in a "primed state," ready for an immediate onset of elongation.

A key element in the regulation of transcription is the organization of DNA into nucleosomes, short segments of DNA (~150 bp) wrapped around octamers of histone proteins (Noll, 1974). Nucleosomes hinder gene transcription in a physical fashion; they render DNA poorly accessible to transcription factors and they prevent movement of the polymerase. Nucleosome positioning is determined by posttranslational modifications of the core histones (trough acetylation, crotonylation, methylation, phosphorylation, SUMOylation, and ubiquitylation) and, to a much lesser extent, by the intrinsic properties of the DNA sequence. Return to Chapter 2, "An Introduction to Signal Transduction," Section "Acetylation, crotonylation, and methylation" (Figure 2-22). This means that control of transcription involves the recruitment of histone-modifying enzymes that render the DNA more (activation of transcription) or less accessible (repression). Here is where the coactivators or corepressors come into play. They are plentiful and among them we count adaptor proteins such as CRTCs, histone acetyltransferases (HAT) (CREBBP, EP300), histone deacetylases (HDAC1, -2, -3), the mediator complex, the chromatin remodeling complex of the SWI/SNF family, the DNA-loop protein cohesin, as well as the previously mentioned super elongation complex. As a consequence of all this, genes can occur in a fully repressed state (characterized by methylation of the DNA and of histone H3 at K9me3 and K27me3), a repressed but poised-for-activation state (characterized by methylation of histone H3 at K4me1) and a transcriptionally active state (characterized by acetylation of histone H3 at K27Ac in the enhancer region and methylation of histone H3 at K4me3 at the promoter element and K79me2 and K36me3 downstream in the DNA coding region) (Figure 7-11). It is important to realize that the transcriptome of cells does not show a distinct boundary between active and repressed genes, but rather shows a broad distribution of RNA levels that ranges from less than one copy of RNA to millions of copies/gene/cell, and it is not clear what level is functionally sufficient.

REVIEW ARTICLES ON GENE TRANSCRIPTION

- Taatjes, D.J., Marr, M.T., Tjian, R., 2004. Regulatory diversity among metazoan co-activator complexes. Nat. Rev. Mol. Cell Biol. 5, 403–410.
- Ptashne, M., 2005. Regulation of transcription: from lambda to eukaryotes. Trends Biochem. Sci. 30, 275–279.
- Ruthenberg, A.J., Li, H., Patel, D.J., Allis, C.D., 2007. Multivalent engagement of chromatin modifications by linked binding modules. Nat. Rev. Mol. Cell Biol. 8, 983–994.
- Lee, T.I., Young, R.A., 2013. Transcriptional regulation and its mis-regulation in disease. Cell 152, 1237–1251.
- Levine, M., Cattoglio, C., Tjian, R., 2014. Looping back to leap forward: transcription enters a new era. Cell 157, 13–25.
- Smith, E., Shilatifard, A., 2013. Transcription elongation checkpoint control in development and disease. Genes Dev. 27, 1079–1088.

Ser133-PHOSPHORYLATED CREB RECRUITS COACTIVATORS CREBBP, PE300, AND CRTC2

The regulatory transcription factor CREB recruits coactivators which, in turn, will modify histones and recruit core-promoter factors, thus giving rise to the constitution of a transcription PIC. The KID domain of serine-133-phosphorylated CREB1 binds to the KIX-domain of CREBBP or EP300 (Figure 7-9). Both are big proteins (2400 aa), with 58% sequence identity, and characterized by eight protein interaction domains, among which a quasi-identical KIX-domain and a histone acetyltransferase (HAT) domain. They acetylate all histones but in particular histone H3 at lys-27 (H3K27Ac is a hallmark of an active enhancer region). Besides histones, both CREBBP and EP300 target other proteins. Important in this context is acetylation of the CREB-regulated coactivator-2, CRTC2. When acetylated on lys-629 it is protected against ubiquitylation, which normally occurs on the same lysine, and thus prevents its destruction by the proteasome (in other words, acetylation augments the protein half-life).

CRTC2 is the second coactivator in the scheme (Figure 7-12) (Lourgenko et al., 2003). In fed (glucose-rich) conditions, it is phosphorylated (on residues ser-171 and ser-274) and sequestered in the cytoplasm through interaction with the 14-3-3β or -γ proteins (YWHAB or -G). These proteins occur in dimers in the cytoplasm and specifically recognize phosphoserine or -threonine motifs. Nuclear localization of CRTC2 thus requires dephosphorylation and in the context of gluconeogenesis this is achieved by inhibiting the SIK2 protein kinase. SIK2 is a substrate of PKA which phosphorylates (on ser-587) and inhibits the enzyme. The loss of active SIK2 causes a gradual dephosphorylation of the phospho-CRTC2 by either the Ca^{2+}-calmodulin-sensitive serine/threonine phosphatase calcineurin (PPP3CA/CNA) or the serine/threonine protein phosphatase PPP4C/PPP4R1 (SMEK) (Figure 7-12) (Wang et al., 2012b; Youn et al., 2010). SIK2, salt-inducible kinase-2, has its name because it was first cloned from the adrenal glands of rats fed with a high salt diet. It is a member of the adenosine monophosphate (AMP)-activated protein kinases. These are protein kinases that operate in carbon metabolic pathways and come into action when ATP levels drop and, as a consequence, 5′-AMP levels rise (Hardie, 2007) (5′-AMP should not by confused with 3′-5′-cyclic AMP). (More information about AMP kinases is given in Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases.”) Dephosphorylated CRTC2 translocates to the nucleus where it binds the zipper region of CREB1, in the vicinity of arg-314. One CREB1-dimer recruits four CRTC2 proteins (Figure 7-12). CRTC2 facilitates the binding of TFIID-associated protein-4 (TAF4) to CREB1 and together they connect with the core-promoter complex TFIID. Once the PIC is composed, transcription starts for a number of genes involved in the gluconeogenic program.

Web resources

For more information about protein domains and their role in cellular processes, we refer to:

- Cell Signaling Technology protein domains and interactions http://www.cellsignal.com/common/content/content.jsp?id=protein-domains
- The Samuel Lunenfeld Research Institute protein interaction domains Website http://pawsonlab.mshri.on.ca/index.php?option=com_content&task=view&id=30&Itemid=63

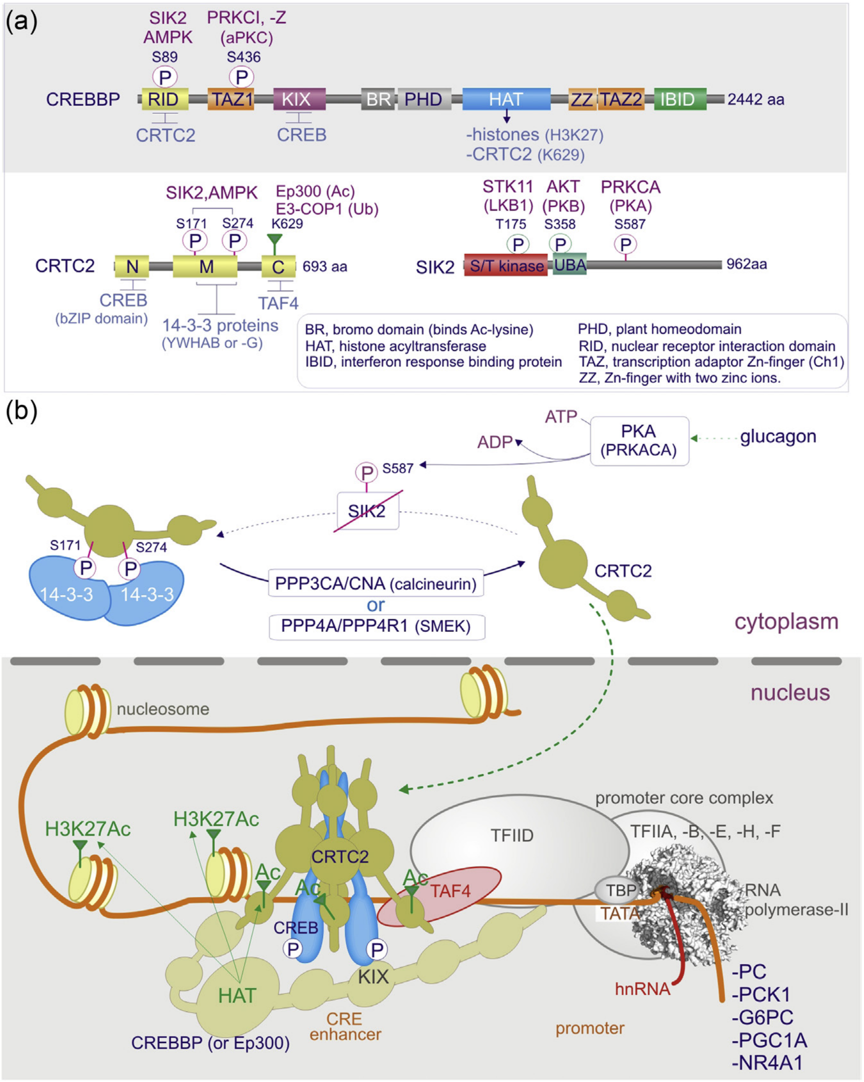


FIGURE 7-12 CREB1-mediated assembly of a pre-initiation transcription complex that drives expression of enzymes involved in the gluconeogenesis pathway. (a) Domain architecture of SIK2, CREBBP, and CRTC2 (b) Glucagon-mediated activation of protein kinase A leads to phosphorylation (on ser-587) and inhibition of the serine/threonine protein kinase SIK2. Because of this, the transcription coactivator CRTC2 will occur mainly in a dephosphorylated state (not sequestered by 14-3-3) and translocates into the nucleus. There it binds phospho-CREB associated with CREBBP (or EP300). Acetylation of CRTC2, on lys-629, by CREBBP or EP300 protects the protein against ubiquitylation (which normally occurs on the same residue). Both proteins will also acetylate histone H3 on lys-27, typical of transcriptionally active enhancer regions. CRTC2 then interacts with TAF4 and brings the core-promoter complex TFIID to the promoter region. TFIID in turn recruits TFIIA, -B, etc., together with RNA polymerase-II. Mediator will also be recruited (not shown). A complete pre-initiation complex is put into place and gene transcription can start for several genes that make up the gluconeogenic program (PC, PCK1, G6PC, PGC1A, and NR4A1).

CREB STIMULATES THE GLUCONEOGENIC PROGRAM

The gluconeogenic genes that are directly activated by CREB1 are PC, PCK1, and glucose-6-phosphatases (G6PC). These are rate-limiting enzymes and increased production enhances gluconeogenesis (return to Figure 7-2). But CREB1 also enhances transcription of PPARGC1A, initially discovered as a coactivator of the nuclear PPARγ receptor, and of NR4A1, an orphan nuclear receptor (Figure 7-13). These should be considered as feed-forward mediators whose expression plays a key role in prolonged fasting. PPARGC1A binds and enhances transcription activity of the glucocorticoid receptor (NR3C1), the HNF4A nuclear receptor, and the transcription factor FOXO1. Collectively, these further enhance expression of PCK1, G6PC, and the glucose transporter GLUT2. NR4A1 enhances expression of the previously mentioned G6PC, GLUT2, and of FBP1 and FBP2. The feed-forward mechanism is all the more essential in view of the finding that CRTC2 escapes ubiquitylation only for a short time. It is prone to deacetylation by the nuclear deacetylase Sirtuin 1 (SIRT1). Once the lys-629 is deacetylated, it becomes the target of the ubiquitylation enzyme E3-COP1. Somehow, the loss of CRTC2 is compensated by the newly recruited transcription factors.

GLUCAGON AND CORTISOL (GLUCOCORTICOID) COOPERATE

Cortisol is a steroid hormone that binds to the glucocorticoid nuclear receptor. The name glucocorticoid derives from its role in glucose metabolism, its synthesis in the cortex of the adrenal gland, and its steroidal structure (cholesterol-based). As mentioned earlier, fasting causes its release and it contributes to gluconeogenesis by enhancing the effect of CREB1 in the liver. Cortisol occupies and stimulates NR3C1, which then can bind PPARGC1A and thus plays an essential role in the previously described feed-forward action of CREB1 (Figure 7-13). Cortisol also plays a key role in the mobilization of glutamine and alanine from striated muscle (metabolites that feed the gluconeogenesis pathway); it causes inhibition of glucose uptake by adipose tissue and it stimulates the breakdown of triglycerides into glycerol and fatty acids (to feed both the gluconeogenic and the ketogenic pathway). We refer to Chapter 8, "Nuclear Receptors," for more information about the glucocorticoid receptor, with special emphasis on its role in inflammation and learning.

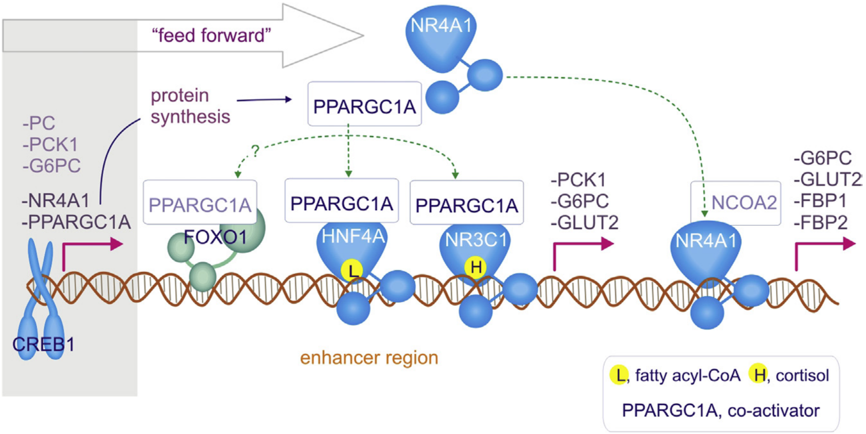


FIGURE 7-13 CREB stimulates a prolonged gluconeogenic program. Among the genes induced by CREB1 are the nuclear receptor NR4A1 (orphan) and the coactivator of nuclear receptors named PPARGC1A. After translation, NR4A1 binds the promoter region of a number of genes involved in gluconeogenesis. PPARGC1A acts as a coactivator of the nuclear receptors HNF4A (when occupied by fatty acyl-CoA) and NR3C1 (when occupied by cortisol). It also binds the transcription factor FOXO1 and again, collectively they further enhance expression of a number of genes involved in gluconeogenesis. These proteins play an essential role when fasting prolongs.

INSULIN CAUSES DISASSEMBLY OF THE CREB-MEDIATED PIC

The arrival of glucose in the blood reduces the release of glucagon and enhances that of insulin in the endocrine pancreas. As a consequence the above-described transcription factor complexes dissociate, the expression of genes involved in the gluconeogenic program attenuates, and glycogen synthase activity is enhanced. Use Figure 7-14 to guide you through the following steps. The liver carries the insulin receptor (INSR) and its occupation gives rise (1) to the activation of AKT1 (PKBα). In contrast to PKA, its phosphorylation of SIK2, on ser-358, leads to enhanced enzymatic activity. Two phosphorylation events are distinguished downstream of SIK2. The first occurs in the cytoplasm (2) and targets CRTC2 and the second occurs in the nucleus (3) and concerns the coactivator EP300 (we ignore how SIK2 reaches the nucleus) (Yang et al., 2001). Phosphorylation of CRTC2, on ser-171 and -274, causes its sequestration by 14-3-3 proteins (YWHA). EP300 is phosphorylated on ser-89 (not conserved in CREBBP) and this causes the disruption of the interaction with CRTC2. Because of this CRTC2 is no longer acetylated by EP300 (4) deacetylation by SIRT1, prevails. The de-acetylated CRTC2

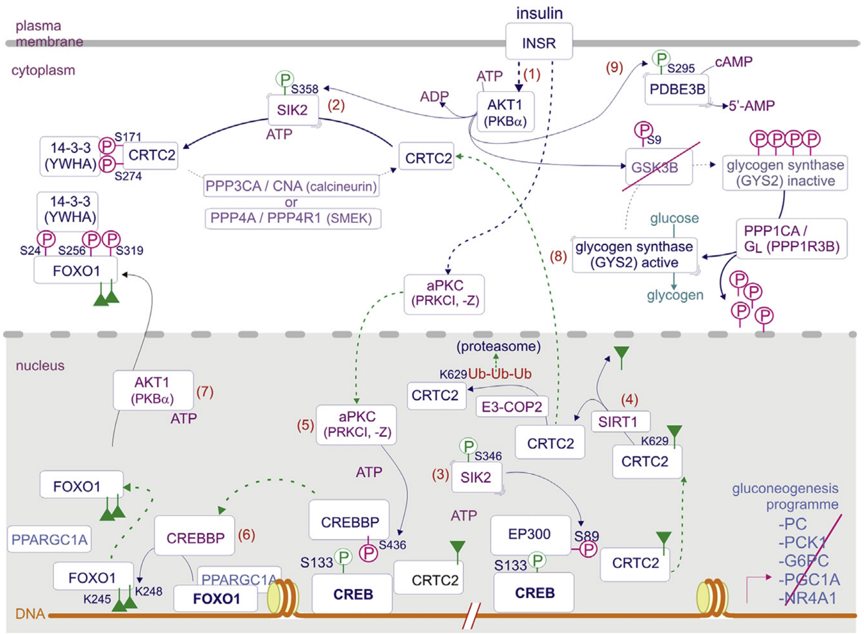


FIGURE 7-14 Insulin arrests the gluconeogenic program. Insulin causes dissolution of a number of transcription factor complexes, involving CREB and FOXO1, and this heralds the attenuation of the gluconeogenic program. Insulin also reactivates glycogen synthase and facilitates the degradation of cAMP through activation of phosphodiesterase-3B.The different processes, indicated by numbers (1), (2), etc., are described in the text.

can be phosphorylated by SIK2, on ser-171 and -274, and this leads to its sequestration by the earlier mentioned 14-3-3 proteins (YWHAB or -G). As an alternative, it becomes a target of the E3-ubiquitin-ligase COP2 and the protein will end up inside the proteasome (to be chopped up in short peptide fragments). Insulin also brings about (5) the phosphorylation of CREBBP on ser-436 (not conserved in EP300) in the nucleus. This involves an atypical protein kinase C (PRKCI or -Z) and leads to the abrogation of the interaction with phospho-CREB (Zhou et al., 2004; He et al., 2009). The now exposed phosphoserine-133 becomes the target of the serine/threonine phosphatase PPP1CA or PPP2CA (Hagiwara et al., 1992; Wadzinski et al., 1993). Lastly, but importantly, the insulin-signaling pathway causes acetylation (6) of FOXO1 through CREBBP. This occurs not by activation of CREBBP but by the prevention of the entry of the histone deacetylase enzymes HDAC4, -5, or -7 into the nucleus (mechanism not shown). Acetylation of FOXO1 disrupts the binding to DNA; hence it disrupts the previously mentioned CREB-mediated feed-forward action described in Figure 7-13. The detached FOXO1 is then (7) phosphorylated by AKT1 (PKBα) and as a consequence, like for CRTC2, the protein finds itself sequestrated by a member of the 14-3-3 family (Mihaylova and Shaw, 2013).

Apart from dissolution of the transcription factor complexes insulin also stimulates (8) glycogen synthase (GYS2) by inactivating the protein kinase GSK3B through phosphorylation by AKT1 (PKBα). GSK3B normally phosphorylates four residues on glycogen synthase and these lead to its inactivation. Glycogen synthase is also a target of PKA. Dephosphorylation and activation of GYS2 is brought about by the serine/threonine phosphatase PPP1CA (GL). This phosphatase is anchored to liver glycogen by a specific regulatory subunit PPP1R3B (initially named GL) (return to chapter 2, figure 2-51).

The production of cAMP diminishes in the absence of glucagon and the presence of insulin. This is the consequence of both a reduced production (loss of glucagon-mediated activation of adenylyl cyclase) and an increased degradation by the cyclic nucleotide phosphodiesterase-3B (PDE3B). The phosphodiesterase (9) is activated by AKT1 via phosphorylation on ser-295 (Ahmed et al., 2007). Loss of cAMP also diminishes the activity of glycogen phosphorylase (enzyme responsible for the breakdown of glycogen). It is active when phosphorylated on serine-14 and dominance of the phosphatase PPPICA/PPP1R3B (GL) leads to its inactivation. A final point, AKT1 also stimulates the translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane. This enables the muscles to reestablish their glycogen stocks. For details about glycogen phosphorylase we refer to Chapter 2, “An Introduction to Signal Transduction,” Section “Putting the components together: the adrenaline to glycogen-phosphorylase signaling pathway” (Figure 2-17).

SIRT1 AND ITS ROLE IN ATTENUATING THE GLUCONEOGENIC PROGRAM AFTER TWO DAYS OF FASTING

Gluconeogenesis takes off strongly in the first two days of fasting and then attenuates in order to prevent an excess of muscle wasting (return to Figure 7-3). Instead, the liver starts producing ketone bodies from glycerol (usage of fatty acids rather than protein). Attenuation of the gluconeogenic program occurs through an enhanced activity of the protein deacetylase SIRT1. It is not clear how this enzyme is regulated, but the AMP-sensitive protein kinase AMPK plays a role (Liu et al., 2008). It has also been shown that AMPK phosphorylates and represses the transcriptional activation of the nuclear receptor HNF4A and this too, like for the loss of nuclear FOXO1 discussed above, abrogates the feed-forward action of CREB (Hong et al., 2003).

DIABETES AND ENHANCED GLUCONEOGENESIS

In diabetes, when insulin is absent (type I) or when it is not functional due to resistance of the target tissues (type II), glucose levels rise to 7 mmol/l and above (state of hyperglycemia). The incidence of type II diabetes is associated with obesity and aging. Chronic hyperglycemia produces organ damage over a period of years and among the consequences we cite retinopathy, neuropathy (loss of sensations in feet and legs), cardiomyopathy, atherosclerosis, and kidney damage. It is not clear to us how an excess of glucose causes tissue dysfunctioning, but aberrant enzymatic and nonenzymatic glycosylation of protein and lipids (both extra- and intracellular) are factors. Moreover, elevated levels of glucose enhance the release of inflammatory cytokines (TNFα, IL1β, IL, and others) by white blood cells (monocytes, macrophages) (Aronson, 2008). The lack of insulin or insulin resistance perturbs glucose homeostasis at two levels. First, it prevents an efficient glucose uptake in organs just after a meal, resulting in a spill over in the kidneys and giving rise to honey-tasting polyuria (hence the name diabetes mellitus). Second, it abrogates the inhibitory control of the above described gluconeogenic program, resulting in a constant high production of glucose by liver and kidneys. Myopathy is one of the consequences because of an increased demand of glutamine and alanine, provided by skeletal muscle (Sandri, 2010).

A current treatment of type II diabetes involves metformin, a biguanide compound that attenuates the gluconeogenic program by inhibiting the production of ATP through oxidative phosphorylation (it interferes with the electron transport chain in mitochondria). This raises the levels of intracellular 5′-AMP which in turn stimulates the protein kinase AMPK, inhibits adenylyl cyclase (attenuating the glucagon-mediated activation of PKA), and has a direct inhibitory effect on fructose-1,6-bisphosphate phosphatase (return to Figure 7-2). AMPK in turn activates atypical PKC (PRKCI or -Z), resulting in the phosphorylation of CREBBP on ser-436 and causing its dissociation from CREB (return to Figure 7-14) (He et al., 2009). Metformin also reduces intestinal absorption of glucose and improves insulin sensitivity so that blood glucose enters the tissues more efficiently (Rena et al., 2013).

References

Ahmed, F., Lindh, R., Tang, Y., Westron, M., Degerman, E., Manganiello, V.C., 2007. Insulin-induced formation of macromolecular complexes involved in activation of cyclic nucleotide phosphodiesterase 3B (PDE3B) and its interaction with PKB. Biochem. J. 404, 257–268.

Akimoto, M., Selvaratnam, R., McNicholl, E.T., Verma, G., Taylor, S.S., 2013. Signaling through dynamic linkers as revealed by PKA. Proc. Natl. Acad. Sci. U.S.A. 110, 14231–14236.

Altarejos, J.Y., Montminy, M., 2011. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat. Rev. Mol. Cell Biol. 12, 141–151.

Amano, T., Sagai, T., Tanabe, H., Mizushina, Y., Nakazawa, H., Shiroishi, T., 2009. Chromosomal dynamics at the Shh locus: limb bud-specific differential regulation of competence and active transcription. Dev. Cell 16, 47–57.

Aronson, D., 2008. Hyperglycemia and the pathobiology of diabetic complications. Adv. Cardiol. 45, 1–16.

Bernstein, B.E., Birney, E., Dunham, I., Green, E.D., Gunter, C., Snyder, M., 2012. ENCODE project consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74.

Cahill, G.F., 2006. Fuel metabolism in starvation. Ann. Rev. Nutr. 26, 1–22.

Cavalli, G., Misteli, T., 2013. Functional implications of genome topology. Nat. Struct. Mol. Biol. 20, 290–299.

Chandramouli, V., Ekberg, K., Schumann, W., Kalhan, S., Wahren, J., Landau, B., 1997. Quantifying gluconeogenesis during fasting. Am. J. Physiol. 273, E1209–E1215.

Colledge, M., Dean, R.A., Scott, G.K., Langeberg, L.K., Huganir, R.L., Scott, J.D., 2000. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 27, 107–119.

Comb, M., Birnberg, N.C., Seasholtz, A., Herrbert, E., Goodman, H.M., 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353–356.

Crick, F., 1970. Central dogma of molecular biology. Nature 227, 561–563.

De Cesare, D., Fimiaa, G.M., Sassone-Corsi, P., 1999. Signalling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem. Sci. 24, 281–285.

Dettori, R., Sonzogni, S., Meyer, L., Lopez-Garcia, L.A., Morrice, N.A., Zeuzem, S., Engel, M., Piiper, A., Neimanis, S., Frodin, M., Biondi, R.M., 2009. Regulation of the interaction between protein kinase C-related protein kinase 2 (PRK2) and its upstream kinase, 3-phosphoinositide-dependent protein kinase 1 (PDK1). J. Biol. Chem. 284, 30318–30327.

Doskeland, S.O., Maronde, E., Gjertsen, B.T., 1993. The genetic subtypes of cAMP-dependent protein kinase – functionally different or redundant? Biochim. Biophys. Acta 1178, 249–258.

El Bacha, T., Luz, M., Da Poian, A., 2010. Dynamic adaptation of nutrient utilization in humans. Nat. Educ. 3, 8.

Esteban, J.A., Shi, S.-H., Wilson, C., Nuiya, M., Huganir, R.L., Malinow, R., 2003. PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nat. Neurosci. 6, 136–143.

Gerich, J.E., Meyer, C., Woerle, H.J., Stumvoll, M., 2001. Renal gluconeogenesis. Diabetes Care 24, 382–391.

Gold, M., 2012. A frontier in the understanding of synaptic plasticity: solving the structure of the postsynaptic density. Bioessays 34, 599–608.

Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., Montminy, M., 1992. Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. Cell 70, 105–113.

Hagiwara, M., Brindle, P., Haroutunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., Montminy, M.R., 1993. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. Mol. Cell Biol. 13, 4852–4859.

Hardie, G.D., 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat. Rev. Mol. Cell Biol. 8, 774–785.

Harootunian, A.T., Adams, S.R., Wen, W., Meiknkoth, J.L., Taylor, S.S., Tsien, R.Y., 1993. Movement of the free catalytic subunit of cAMP-dependent protein kinase into and out of the nucleus can be explained by diffusion. Mol. Biol. Cell 4, 993–1002.

He, L., Sabet, A., Djedjos, S., Miller, R., Sun, X., Hussain, M.A., Radovick, S., Wondisford, F.E., 2009. Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. Cell 137, 635–646.

Holmberg, J., Perlmann, T., 2012. Maintaining differentiated cellular identity. Nat. Rev. Genet. 13, 429–439.

Hong, Y.H., Varanasi, U.S., Yang, W., Leff, T., 2003. AMP-activated protein kinase regulates HNF4alpha transcriptional activity by inhibiting dimer formation and decreasing protein stability. J. Biol. Chem. 278, 27495–27501.

Hornbeck, P.V., Kornhauser, J.M., Tkachev, S., Zhang, B., Skrzypek, E., Murray, B., Latham, V., Sullivan, M., 2012. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res. 40, D261–D270.

Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, A., Schmid, W., Beerman, F., Schutz, G., 1994. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc. Natl. Acad. Sci. U.S.A. 91, 5647–5651.

Iguchi-Ariga, S.M., Schnaffer, W., 1989. CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTA abolishes specific factor binding as well as transcriptional activation. Genes Dev. 3, 612–619.

Impey, S., McCorkle, S.R., Cha-Molstad, H., Dwyer, J.M., Yochum, G.S., Boss, J.M., McWeeney, S., Dunn, J.J., Mandel, G., Goodman, R.H., 2004. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119, 1041–1054.

Jacob, F., Monod, J., 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3, 318–356.

Jacob, F., Perrin, D., Sanchez, C., Monod, J., 1960. L'opéron: un groupe de gènes à expression coordonnée par un opérateur. C. R. Hebd. Acad. Sci. 250, 1727–1729.

Kannan, N., Haste, N., Taylor, S.S., Neuwald, A.F., 2007. The hallmark of AGC kinase functional divergence is its C-terminal tail, a cis-acting regulatory module. Proc. Natl. Acad. Sci. U.S.A. 104, 1272–1277.

Koth, C.M., Murray, J.M., Mukund, S., Madjidi, A., Minn, A., Clarke, H.J., Wong, T., Chiang, V., Luis, E., Estevez, A., Rondon, J., Zhang, Y., Hötzel, I., Allan, B.B., 2012. Molecular basis for negative regulation of the glucagon receptor. Proc. Natl. Acad. Sci. U.S.A. 109, 14393–14398.

Lee, T.I., Young, R.A., 2013a. Transcriptional regulation and its misregulation in disease. Cell 152, 1237–1250.

Lee, T.I., Young, R.A., 2013b. Transcriptional regulation and its misregulation in disease. Cell 152, 1237–1251.

Levine, M., Cattoglio, C., Tjian, R., 2014. Looping back to leap forward: transcription enters a new era. Cell 157, 13–25.

Liu, Y., Dentin, R., Chen, D., Hedrick, S., Ravnskjaer, K., Schennk, S., Llne, J., Leyers, D.J., Cole, P., Yates, J., Olefsky, J., Guarente, L., Montminy, M., 2008. A fasting inducible switch modulates gluconeogenesis via activator/co-activator exchange. Nature 456, 269–273.

Lourgenko, V., Zhang, W., Mickanin, C., Daly, I., Jian, C., Hexham, J.M., et al., 2003. Identification of a family of cAMP response element-binding protein co-activators by genome-scale analysis in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 100, 12147–12152.

Lüscher, C., Nicoll, R.A., Malenka, R.C., Muller, D., 2000. Synaptic plasticity and dynamic modulation of the postsynaptic membrane. Nat. Neurosci. 3, 545–550.

Mihaylova, M.M., Shaw, R.J., 2013. Metabolic reprogramming by class I and II histone deacetylases. Trends Endocrinol. Metab. 24, 48–57.

Montminy, M.R., Bilezikjian, L.M., 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328, 175–178.

Montminy, M.R., Searino, K.A., Wagner, J.A., Mandel, G., Goodman, R.H., 1986. Identification of a cyclic-AMP responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. U.S.A. 83, 6682–6686.

Noll, M., 1974. Subunit structure of chromatin. Nature 251, 249–251.

Oliveria, S.F., Dell'Acqua, M.L., Sather, W.A., 2007. AKAP 79/150 anchoring of calcineurin controls neuronal L-type Ca^{2+} channel activity and nuclear signalling. Neuron 55, 261–275.

Parker, D., Ferreri, K., Nakajima, T., LaMorte, V.J., Evans, R., Koerber, S.C., Hoeger, C., Montminy, M.R., 1996. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. Mol. Cell Biol. 16, 694–703.

Radhakrishan, I., Perez-Alvarado, G.C., Parker, D., Dyson, H.J., Montminy, M.R., Wright, P.E., 1997. Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:co-activator interactions. Cell 91, 741–752.

Rena, G., Pearson, E.R., Sakamoto, K., 2013. Molecular mechanism of action of metformin: old or new insights? Diabetologica 56, 1898–1906.

Sample, V., DiPilato, L.M., Yang, J.H., Ni, Q., Saucerman, J.J., Zhang, J., 2012. Regulation of nuclear PKA revealed by spatiotemporal manipulation of cyclic AMP. Nat. Chem. Biol. 8, 375–382.

Sanderslon, J.L., Dell'Acqua, M.L., 2011. AKAP signaling complexes in regulation of excitatory synaptic plasticity. Neuroscientist 17, 321–326.

Sandri, M., 2010. Autophagy in skeletal muscle. FEBS 584, 1411–1416.

Schumacher, M.A., Goodman, R.H., Brennan, R.G., 2000. The structure of a CREB bZIP. somatostatin CRE complex reveals the basis for selective dimerization and divalent cation-enhanced DNA binding. J. Biol. Chem. 45, 35242–35247.

Scott, J., Dessauer, C.W., Tasken, K., 2013. Creating order from chaos: cellular regulation by kinase anchoring. Annu. Rev. Pharmacol. Toxicol. 53, 187–210.

Siu, F.Y., He, M., de Graaf, C., Han, G.W., Yang, D., Zhang, Z., Zhou, C., Xu, Q., Wacker, D., Joseph, J.S., Liu, W., Lau, J., Cherezov, V., Katritch, V., Wang, M.W., Stevens, R.C., 2013. Structure of the human glucagon class B G-protein-coupled receptor. Nature 499, 444–451.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.

Taylor, S.S., Ilouz, R., Zhang, P., Kornev, A.P., 2012. Assembly of allosteric macromolecular switches: lessons from PKA. Nat. Rev. Mol. Cell Biol. 13, 646–658.

Theunissen, T.W., Jaenisch, R., 2014. Molecular control of induced pluripotency. Cell Stem Cell 14, 720–734.

Wadzinski, B.E., Wheat, W.H., Jaspers, S., Peruski, L.F., Lickteig, R., Johnson, G.L., Klemm, D.J., 1993. Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. Mol. Cell Biol. 13, 2822–2834.

Wang, X., Bai, L., Bryant, G.O., Ptashne, M., 2011. Nucleosomes and the accessibility problem. Trends Genet. 12, 487–492.

Wang, M., Weiss, M., Simonovic, M., Haertinger, G., Schrifmpf, S.P., Hengartner, M.O., von Mering, C., 2012. PaxDb, a database of protein abundance averages across all three domains of life. Mol. Cell Proteomics 11, 492–500.

Wang, Y., Li, G., Goode, J., Paz, J.C., Ouyang, K., Screaton, R., Fischerr, W.H., Chen, J., Tabas, I., Montminy, M.R., 2012. Inositol-1,4,5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes. Nature 485, 128–132.

Wernig, L., Meissnerr, A., Foreman, R., Brambrink, T., Ku, M., Hochedlingerr, J., Bernstein, B.E., Jaenisch, R., 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448, 318–324.

Willoughby, D., Masada, N., Wachten, S., Pagano, M., Halls, M.L., Everett, K.L., Ciruela, A., Cooper, D.M.F., 2010. AKAP79/150 interacts with AC8 and regulates Ca^{2+}-dependent cAMP synthesis in pancreatic and neuronal systems. J. Biol. Chem. 285, 20328–20342.

Wise, J.K., Hendler, R., Felig, P., 1973. Influence of glucocorticoids on glucagon secretion and plasma amino-acid concentrations in man. J. Clin. Invest. 52, 2774–2782.

Wu, J., Brown, S.H., von Daake, S., Taylor, S.S., 2007. PKA type IIa holoenzyme reveals a combinatorial strategy for isoform diversity. Science 318, 274–279.

Yang, W., Hong, Y.H., Shen, X.Q., Frankowski, C., Camp, H.S., Leff, T., 2001. Regulation of transcription by AMP-activated protein kinase: phosphorylation of p300 blocks its interaction with nuclear receptors. J. Biol. Chem. 276, 38341–38344.

Youn, Y.-S., Lee, M.-W., Ryu, D., Kim, J.H., Ma, H., Seo, W.-Y., Kim, Y.-N., Kim, S.S., Lee, C.H., Hunterr, T., Choi, C.S., Montminy, M.R., Koo, S.-H., 2010. Suppressor of MEK null (SMEK)/protein phosphatase 4 catalytic subunit (PP4C) is a key regulator of hepatic gluconeogenesis. Proc. Natl. Acad. Sci. U.S.A. 107, 17704–17709.

Zhang, D.X., Glass, C.K., 2013. Towards an understanding of cell-specific functions of signal-dependent transcription factors. J. Mol. Endocrinol. 51, T37–T50.

Zhang, P., Smith-Nugyen, E.V., Keshwani, M.M., Deal, M.S., Kornev, A.P., Taylor, S.S., 2012. Structure and allostery of the PKA RIIβ tetrameric holoenzyme. Science 335, 712–716.

Zhou, X.Y., Shibusawa, N., Naiik, K., Porras, D., Temple, K., Ou, H., Kaihara, K., Roe, M.W., Brady, M.J., Wondisford, F.E., 2004. Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB binding protein. Nat. Med. 10, 633–637.

CHAPTER

8

Nuclear Receptors

In this chapter we mainly deal with hydrophobic first messengers, molecules that partition badly in water because they offer very little electrostatic interactions. They require carrier proteins (globulins) for their distribution, they can enter the plasma membrane, and they are captured by intracellular receptors. These receptors are DNA-binding proteins and act as transcription factors, hence their qualification as "nuclear receptors." Some of the nuclear receptors, in particular those of steroid hormones and vitamin D, additionally signal in a non-genomic fashion; activating signal transduction pathways common to transmembrane receptors. As there are always exceptions to a rule, the steroid hormone progesterone also binds to and activates a plasma-membrane Ca^{2+} channel in spermatozoa, thereby controlling motility and the acrosome reaction.

STEROID HORMONES

Interest for sex steroids has a very long history going back to the origins of our great affair with domestic animals (Needham and Lu, 1970). Castration was widely practiced to avoid unwanted reproduction and to facilitate husbandry by making the males more docile (Figure 8-1). Barrows (pig), bullocks (cattle), geldings (horse), and oxen (name for castrated cattle kept for draft purposes) are common to nearly all continents and cultures. Castration was not limited to the husbandry of animals; eunuchs played an important role in the management of the imperial court of medieval China and of the Ottoman Empire (innocently looking after the Sultan's harem). The use of placental extracts gets a first mention in a Chinese pharmacopoeia of 725 AD, though their widespread use had to wait another seven centuries until promulgated by Chu Chen-Heng. The importance of testes was also realized and extracts were prepared and applied in various forms of organotherapy. Chu Chen-Heng encouraged their application for the treatment of many conditions. According to him, "*all people suffering from impotence, sexual debility, excess yang of the burning feverish type which no medicine can benefit, will improve. The natural precipitate of urine can also drive*

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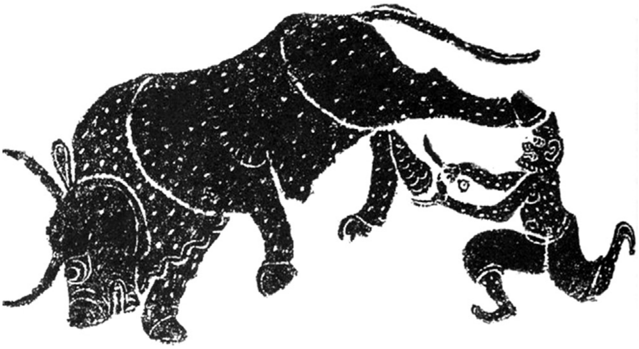


FIGURE 8-1 Bull castration in China as depicted by a tomb mural dating to the Eastern Han period, first to second century AD. *Image courtesy of Roel Sterckx, Joseph Needham, Professor of Chinese History, Science and Civilization, University of Cambridge, UK.*

out the undue fire element affecting the liver and the bladder." More importantly, the Chinese had, over this period of time, developed a remarkable set of extraction and fractionation techniques, with the aim of obtaining active materials from urine. Pharmacists were engaged "almost on a manufacturing scale," using up to 700 l at a time, to prepare active products by precipitation with calcium sulfate (which sedimented steroid conjugates along with protein), evaporation to dryness, sublimation, and crystallization. Joseph Needham had no doubt that the Chinese had, between the eleventh and seventeenth centuries, achieved preparations of androgens and estrogens that were "*probably quite effective in the quasi-empirical therapy of the time*." Surprisingly, nothing of this all ever penetrated the Western scientific community and the discovery of steroid hormones started from scratch in Europe in the second half of the nineteenth century.

The roots of scientific development in China including aspects that we now call endocrinology have been documented by Joseph Needham, a Cambridge biochemist who turned his attention to the science and civilization of China (Needham and Lu, 1970).

Among the scientists who laid the foundation for later discoveries of steroid hormone we are obliged to cite Brown-Séquard who described in 1898 the rejuvenating consequences of the self-injection of testicular extracts (related in Chapter 1, "Prologue"). These effects may or may not have been due to androgenic steroids. Or they may have been a matter of self-delusion ("placebo effect"). Extracts were often made in aqueous environments which must have resulted in very low yields of the hydrophobic steroids. However, his contemporary, the Scottish surgeon George Thomas Beatson undoubtedly observed the regression of inoperable breast tumors following ovariectomy, the female equivalent of castration, and laid the foundation for what we now call hormone therapy for breast cancer (tamoxifen) (Beatson, 1896). Thomas Addison, at Guys Hospital in London, UK, made an important contribution in 1855 with a detailed description of clinical symptoms that he related to the impaired function of the adrenal gland (Addison, 1855). The rare disorder, now known as Addison disease, has been a driving force in the discovery of mineral and glucocorticosteroids. Arnold Berthold, at the George-August University of Göttingen, Germany, demonstrated that reimplantation of testes in castrated male chicken, under the condition where the organs had established a blood connection, restored all the rooster characteristics (Soma, 2006). This finding played an essential role in the discovery of testosterone.

Capons, the name given to castrated chicken, helped Fred Koch and his coworkers, at the University of Chicago, USA, to test extracts of bull testes, and in 1927 they showed that certain fractions caused these birds to re-masculinize. By 1935, Ernest Laqueur, a pharmacologist working at the University of Amsterdam, the Netherlands, had determined the substance's molecular structure and named it testosterone (David et al., 1935). Laqueur financed his research in part by the revenues of the company Organon that he had founded, together with one of his colleagues Jacques van Oss and the meet company director Saal Zwanenberg.

Besides roosters, pregnant women, rabbits, and pigs too have played an important role in the discovery of sex steroids. When Bernard Zondek and Selmar Aschheim, working at the Universitäts-Frauenklinik at the Berlin Charité, Germany, had shown in 1928 that the urine of pregnant women possesses estrogenic activity when injected into immature laboratory mice (Zondek, 1928). Edward Doily decided to stop isolating hormones from animal ovaries and instead started distributing big glass bottles to pregnant family members, friends, and the local obstetrics clinic. In 1930, from hundreds of liters of urine, he purified and characterized the first steroid hormone, the estrous-inducing substance estrone (Doisy et al., 1930). Edward Doily relates that the driver who was hired to make collections of urine once committed a traffic violation. When stopped the policemen noticed the amber-colored gallon-size bottles in the back of the car and was convinced to be dealing with a bootlegger. After pulling a cork and sniffing the content, the cop dismissed the driver by saying "My God, it is urine! Your job is bad enough without getting pinched for it—drive on!" (Doisy, 1976).

At the turn of the twentieth century, the German gynecologist Ludwig Fraenkel, at the University of Breslau (now Wroclaw, Poland) became fascinated by ovaries, and in particular their corpora lutea. Gustav Born, an eminent anatomist, had entrusted to him in the last hours of his life that the corpus luteum could well be a hormonal gland. The idea had come to Born's mind after having observed detailed drawings of mouse ovaries by the famous anatomist Johannes Sobotta (Sobotta/Becher Atlas of Human Anatomy). Brown had noticed the adenoid structures which, to his understanding, were characteristic of ductless glands. Auguste Prenant, at the University of Nancy, France, had come to the same conclusion but apparently failed to convince anybody to follow it up. Fraenkel did and he initially started to remove whole ovaries from rabbits that had copulated a few days previously and found that none was pregnant. It should be noted that rabbits have long periods of estrus (heat) but only ovulate shortly after copulation (within 12 h). He obtained similar results when only removing the corpus luteum. Fraenkel concluded that it played a role in the nidation of the embryo. With the help of Erich Fels and the chemists Karl Slotta and Heinrich Ruschig, he started purifying the "progestational" hormone from pig ovaries. Because Fraenkel enjoyed very little financial support pregnant women helped him with the extraction process in exchange for free medical care at the hospital. In the race against the clock, because the National Socialists threatened to close down the Breslau laboratory, Fraenkel failed to publish his early findings properly (Frobenius, 1998). The first articles on the purification of progesterone were issued almost simultaneously in 1934 from four different laboratories: Breslau (Slotta et al., 1934), Danzig (now Gdansk, Poland) (Butenandt et al., 1934), Rochester (USA) (Wintersteiner and Allen, 1934), and Basel (Switzerland) (Hartmann and Wettstein, 1934). Fraenkel did not author any of these papers but may be considered the spiritual leader of the whole undertaking.

ESTROGEN AND PROGESTERONE

The root of estrogen and related products is odd. It derives from the word oestrous (estrus in American spelling) which in turn comes from the Greek "oistros" meaning a gadfly (also known as horsefly). Fritz Spiegl points out that to be stung by a gadfly was thought to send people, especially women, into a frenzy (Spiegel, 1996).

The origin of the word progesterone is more rational. It was coined collectively by Willard Allen, Adolf Butenandt, George Corner, and Karl Slotta and combines both a physiological and chemical description: a **proge**stational **ster**eroidal ket**one**. Initial names were progestin and luteosterone.

Purification of hormones from the adrenal gland, other than adrenaline, also had started at the turn of the twentieth century, but progress was slower. Perhaps there was a lesser sense of urgency because apart from a potential remedy for Addison disease few therapeutic applications for adrenal gland extracts were envisioned at that time. Early studies had shown that after lipophilic extraction, some of the fractions affected the metabolism of electrolytes (mineral corticoids), others of carbohydrates and proteins (glucocorticoids). It was also found that adrenalectomized animals were more susceptible to bacterial toxins and that some of the fractions provided relief (anti-inflammatory action of glucocorticoids) but this did not yet spark the interest for large-scale purification efforts. Thomas Reichstein from Basel, Switzerland, brought new impetus in the search when he discovered that these fractions contained steroids. Characterization of a wide range of glucocorticoids, different metabolic products, soon followed both by Reichstein and by Edward Kendall at Rochester, USA. The application of purified cortisone or desoxycorticosterone became a standard to maintain patients with Addison disease in relative good condition. A new turn was given by 1948 when it was shown convincingly by the rheumatologist Philip Hench, in collaboration with Kendall and with steroids produced by Merck and Co, that 17-hydroxy-11-dehydrocorticosterone ("cortisone") relieved joint inflammation in patients with rheumatoid arthritis. Cortisone became widely appreciated as a pharmacological anti-inflammatory agent and not as a hormone of the adrenal cortex (Kendall, 1950). Purification (Grundy et al., 1952) and subsequent molecular characterization of aldosterone, the last of the adrenal hormones, occurred in 1954, by Sylvia Simpson-Tait and James Tait, at the Middlesex Hospital, London, UK, in collaboration with Reichstein (Simpson et al., 1954).

The importance of these discoveries must be clear to everybody. Steroids play a role virtually everywhere and are used in the treatment of chronic inflammatory disorders and bone loss after menopause (estrogen or DHEA), in the treatment of Addison's disease, and in congenital adrenal hyperplasia (glucocorticoids and mineralcorticoids), in the enhancement of athletic performances (testosterone), they are used for contraception (estradiol), and gender change (transsexualism; testosterone or estrogen/progesterone). Blocking the action of steroids by receptor antagonists is applied for certain cancers (breast and prostate) (tamoxifen, flutamide), in medically assisted reproduction (clomifene), in gender change (finasteride), and in the treatment of hypertension (spironolactone).

STEROIDS ACCUMULATE IN THE NUCLEUS

The identification of receptors for the steroid hormones started in the early 1960s with the synthesis of tritium-(^{3}H)-labeled estrogens. Initial studies showed that the label was retained predominantly in the cells of the uterus and the vagina of goats and sheep (Glascock and Hoekstra, 1957). The organ-specific retention provided strong evidence for binding to specific receptors but further proof was required. Noteboom and Gorki demonstrated the accumulation of ^{3}H-estradiol in both the nuclear and cytosolic fractions of myofibrillar cells from the rat uterus (Noteboom and Gorski, 1965). The first actual demonstration of steroid binding to a receptor protein, indeed, the first demonstration of the binding of any hormone to any receptor in a cell-free system, was reported in 1966 by Toft and Gorski (1966). They recovered radioactivity from the nuclear fraction and to a lesser extent from the cytosol. After centrifuging the cytosolic material through a sucrose density gradient, they found the radioactivity in a component that sedimented at ~9 Svedberg (S), equivalent to a protein of around 200 kDa. Binding of the hormone could be antagonized by diethylstilbestrol (DES), but not by the non-estrogenic steroids such as testosterone and corticosterone. Similar high affinity-binding proteins ($K_D \sim 0.1$–10 nM) were found in soluble fractions prepared from homogenates of various unstimulated hormone-responsive tissues. We now know that the 9S fraction comprises the steroid receptor, only 60 kDa, bound to chaperones (see Figure 8-9 "ligand binding with the help of HSP"). Following binding of hormone with time, these receptor proteins accumulated in the nuclear fraction. The key experimental observation was the retention of [^{3}H]-corticosterone in the nuclear fraction of hepatoma cells, at the expense of a loss of radioactivity from the cytosol (Rousseau et al., 1973). The clearest, visually speaking, demonstration that cytoplasmic receptors translocate to the nucleus after exposure to ligand, came from experiments with green fluorescent protein-tagged glucocorticoid receptors. The progress from cytosolic to nuclear localization is illustrated in Figure 8-2. For green fluorescent protein, return to Chapter 6 "Intracellular Calcium," Figure 6-9. Because of these findings steroid-hormone receptors were qualified as nuclear receptors.

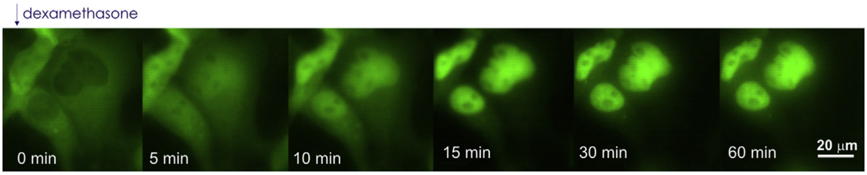


FIGURE 8-2 The progression of nuclear translocation of GFP-tagged glucocorticoid receptors expressed in COS-1 cells following treatment with dexamethasone, a synthetic glucocorticoid. *Image courtesy of M. Kawata (Nishi et al., 1999).*

The ligand-dependent translocation pattern is broadly similar for all steroid hormone receptors which are, besides the already mentioned glucocorticoid (NR3C1) and estrogen receptors (ESR), the mineralocorticoid (NR3C2 or MR), androgen (AR) and progesterone (PGR) receptors. However, the partition of steroid receptors into either the cytosol or the nucleus is never absolute. Their distribution between the nuclear and cytoplasmic compartments is variable and it depends upon cell type, receptor subtype, and possibly the stage of the cell cycle. For instance, unliganded estrogen receptors can remain in the nucleus and although they are transcriptionally "unproductive," they play a role in keeping target genes in a poised (permissive) state (Metivier et al., 2004). As we see later, not only steroids but also retinoic acids, fatty acids, eicosanoids, cholesterol, bile acids, and xenobiotics bind to receptors that predominantly reside in the nucleus (see Figures 8-3 and 8-8). These receptors tend not to shuttle back into the cytoplasm.

STEROIDS REGULATE GENE TRANSCRIPTION

Steroid hormones are for many reasons of historical significance and not in the least because they were the first hormones seen to have a direct effect on gene transcription. This observation was made in insects, more precisely during the process of molting and pupation (metamorphosis). It concerns giant (or polytene) chromosomes from the salivary glands, which throughout the molting process manifest a succession of morphological changes, observed by light microscopy, and described as "puffs." It was then shown that these puffs are regions in the normally highly condensed giant chromosome which loosen up so that the DNA becomes accessible for RNA polymerases. The ensuing transcription process is readily made visible by autoradiography after the addition of ^{3}H-uridine (which incorporates into nascent mRNA) (Figure 8-3) (Pelling, 1959). The succession of puffs, and thus the succession of changes in gene transcription, is under the control of a steroidal hormone produced in the prothoracic gland. It was purified from pupae of male silkworm by Adolf Butenandt and Peter Karlson and named ecdysone. When the purified hormone was injected into Chironomus larvae, Karlson and Ulrich Clever, at the Max Planck Institute, Tübingen, Germany, demonstrated the immediate appearance of a small number of metamorphosis "puffs" (Clever and Karlson, 1960). They concluded that the "puffs" were the consequence of a direct interaction between the hormone and specific genes. This concept has driven steroid hormone research ever since and led to the current understanding that steroid hormone receptors are transcription factors.

In biology, molting is the process in which an insect casts off a part of its body, often the exoskeleton, in order to progress in its life cycle. Molting is an essential event during the process of metamorphosis. It is also known as sloughing, shedding or, for some species, ecdysis (hence, we assume, the name ecdysone).

Giant or polytene chromosomes occur in specialized cells, commonly found in the salivary glands of insects, which appear as a consequence of repeated rounds of DNA replication without subsequent cell division. These chromosomes align and are highly condensed, giving rise to characteristic band patters. The bands on these chromosomes have been studied extensively by Thomas Morgan and contributed to the development of the gene theory in the early twentieth century. This subject is dealt with in Chapter 10, about "notch."

CHAPERONES OF THE HEAT-SHOCK FAMILY

Chromosome puffs helped to discover heat-mediated chaperone protein (HSP) expression. As a young graduate student at the University of Pavia in the early 1960s, Ferruccio Ritossa investigated the type of nucleic acid in the chromosomal puffs of *Drosophila* salivary glands. He used cultured cells, which are normally kept at 25 °C but someone had inadvertently raised the temperature of his incubator to 37 °C. What he noticed was a complete change in the puffing pattern. Somehow, the heat shock had changed the pattern of gene expression. He took advantage of this error and studied the effect of temperature in more detail and confirmed that indeed heat shocks change puffing patterns (Figure 8-3(c)) (Ritossa, 1962). The proteins that were induced by the temperature stress were named heat-shock proteins (HSPs) (Tissieres et al., 1974). Only later it was discovered that these "HSPs" were in fact members of the chaperone protein family which function in diverse cellular roles at "normal" temperatures (folding, multimerization, selection, packaging, secretion, and degradation of proteins) (Hendrick and Hartl, 1993). As we will learn, HSPs play a role in rendering certain nuclear receptors accessible for their steroid ligand.

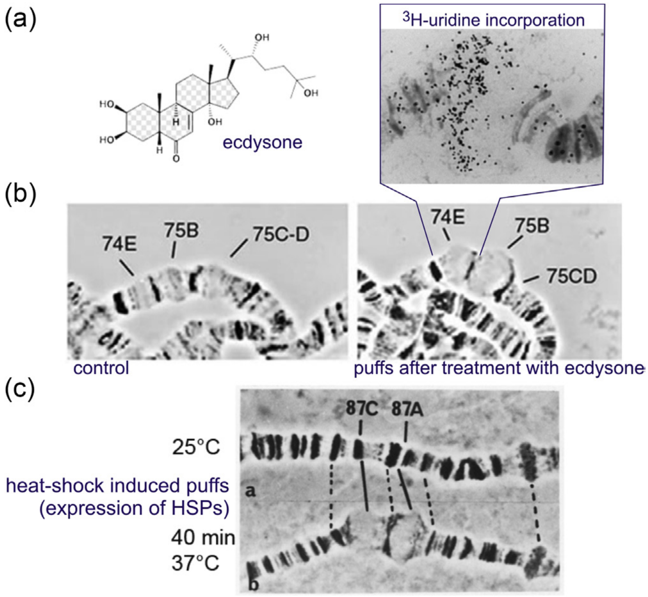


FIGURE 8-3 **The first tangible sign of steroid hormone-mediated gene expression.** (a) Structure of ecdysone, a steroid hormone that plays an important role in the molting process of insects. (b) Treatment of cell extracts from insect (*Drosophila*) salivary glands with ecdysone causes the formation of new puffs on giant chromosomes and the loss of others. Puffs are sites with enhanced gene transcription as witnessed by the high level of incorporation of ^{3}H-uridine (Pelling, 1959). This observation provided early evidence of a role of steroids in the regulation of gene transcription. *Image with permission from Zhimulef et al. (2001)*. (c) As an aside, giant insect chromosomes also revealed for the first time heat-induced changes in gene expression and opened the way for the discovery of protein chaperones (the so-called heat-shock proteins or HSPs). *Image with permission from Ashburner and Bonner (1979).*

A SUPERFAMILY OF NUCLEAR RECEPTORS

The receptors for steroid hormones are members of a very much larger class of ligand-activated transcription factors. It includes the receptors for thyroid hormones, for retinoids (i.e., all-*trans*-retinoic acid or vitamin A, and its isomer 9-*cis*-retinoic acid) and for vitamin D (cholecalciferol). The family also includes receptors for numerous other hydrophobic messengers, some of which exist naturally in the body, like fatty acids, bile acids, oxysterols and eicosanoids, and others that are of external origin, such as certain pharmaceuticals, carcinogens, and environmental pollutants (known collectively as xenobiotics). The classification of the nuclear receptor family, obtained by building phylogenetic trees from sequence information, their ligands, and some of the ligand structures are shown in Figure 8-4. Among the first sequences discovered, in the 1980s, are the human glucocorticoid and estrogen receptors (Hollenberg et al., 1985; Green et al., 1986).

	nuclear receptor (dimer)	**nomenclature**	**ligand** (+ chemical structure)
type 1	thyroid hormone receptor (THR/THR or THR/RXRA)	THRA (NR1A1), α THRB (NR1A2), β	thyroid hormone thyroid hormone
	retinoic acid receptor (RAR/RXR)	RARA (NR1B1), α RARB (NR1B2), β RARG (NR1B3), γ	retinoic acid retinoic acid retinoic acid
	peroxisome proliferator-activated receptor (PPAR/RXR)	PPARA (NR1C1), α PPARD (NR1C2), δ PPARG (NR1C3), γ	fatty acids, leukotriene B4 fatty acids, prostaglandin I_2 fatty acids, prostaglandin D_2
	reverse-ErbA	NR1D1 NR1D2	orphan orphan
	RAR-related orphan receptor	RORA (NR1F1), α RORB (NR1F2), β RORC (NR1F3), γ	cholesterol *all-trans*-retinoic acid *all-trans*-retinoic acid
	liver X receptor (LXR/RXR)	NR1H2 (LXR-β) NR1H3 (LXR-α)	oxysterols oxysterols
	farnesoid receptor (NR1H/RXR)	NR1H4 (FXRα) NR1H5 (FXRβ)	bile acids, fexaramine pseudogene
	vitamin D receptor (VDR/RXR)	VDR (NR1I1)	1,25-dihydroxy vitamine D_3
	pregnane receptor (NR1I2/RXR)	NR1I2 (PXR)	xenobiotics, pregnenolone
	constitutive androstane receptor (NR1I3/RXR)	NR1I3 (CAR)	xenobiotics, phenobarbital
type 2	hepatocyte nuclear factor	HNF4A (NR2A1) HNG4G (NR2A2)	fatty acyl-CoA (linked with diabetes) orphan
	retinoid X receptor (RXR/RXR and heterodimer with numerous partners)	RXRA (NR2B1), α RXRB (NR2B2), β RXRG (NR2B3), γ	*9-cis*-retinoic acid *9-cis*-retinoic acid all-trans-retinoic acid
	testis receptor	NR2C1 (Tr1) NR2C2 (Tr2)	orphan orphan
	tailless	NR2E1 (TLL)	orphan
	photoreceptor-specific NR	NR2E2 (PNR)	orphan
	chicken ovalbumin upstream promotor	NR2F1 (COUP-TF1) NR2F2 (COUP-TF2)	orphan orphan
	ErbA2-related	NR2F6 (EAR2)	orphan
type 3	oestrogen receptor (ESR/ESR)	ESR1 (NR3A1) ESR2 (NR3A2)	oestradiol-17β, tamoxifen oestradiol-17β
	oestrogen receptor-related receptor	ESRRA (NR3B1), α ESRRB (NR3B2), β ESRRG (NR3B3), γ	orphan diethylstilbestrol, 4-OH tamoxifen diethylstilbestrol, 4-OH tamoxifen
	glucocortiocoid receptor (NR3C1/NR3C1 or /NR3C2)	NR3C1 (GR)	cortisol, dexamethasone aldosterone
	mineral corticoid receptor (NR3C2/NR3C2 or /NR3C1)	NR3C2 (MR)	aldosterone, spirolactone, cortisol
	progesterone receptor (PGR/PGR)	PGR (NR3C3)	progesterone, medroxyprogesterone
	androgen receptor (AR/AR)	AR (NR3C4)	testosterone, flutamide
type 0, 4, 5, 6	NGF-induced factor B Nur-related factor neuron-derived orphan steroidogenic factor-1 (monomer) liver receptor homologous germ cell nuclear factor DSS-AHC chromosome X short heterodimeric partner	NR4A1 (NGFIB) NR4A2 (NURR1) NR4A3 (NOR1) NR5A1 (SF1) NR5A2 (LRH1) NR6A1 (GCNF) NR0B1 (DAX1) NR0B2 (SHP)	orphan orphan (linked withParkinsonism) orpha orphan phospholipid, dilauroyl-PC orphan orphan orphan

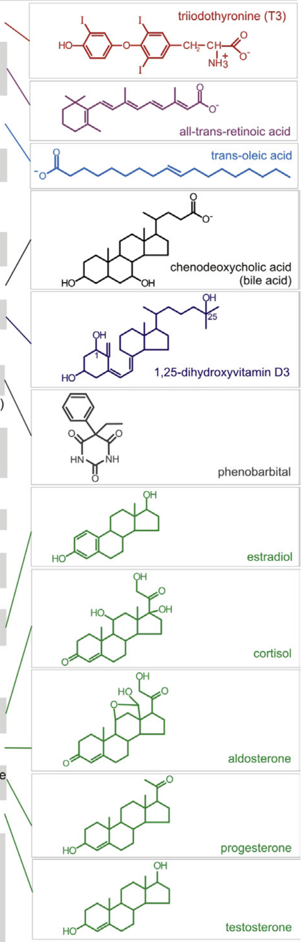


FIGURE 8-4 The nuclear receptor family (NR type 0–6) and some of their ligands. *Image adapted in part from reference Gronemeyer et al. (2004). The nomenclature is according to the HUGO gene nomenclature committee.*

The human family is subdivided into seven types (NR0–6). As you will notice, receptors for similar ligands are not necessarily confined to the same type; examples are RAR (type 1) and RXR (type 2). This lack of correspondence suggests that the receptors have arisen from an ancestral orphan receptor (lacking a ligand) and that the acquisition of the capacity to bind particular ligands may have arisen independently and at different times during evolution (Laudet, 1997).

Nuclear receptors have a wide range of biological functions including the regulation of growth and embryonic development, the maintenance

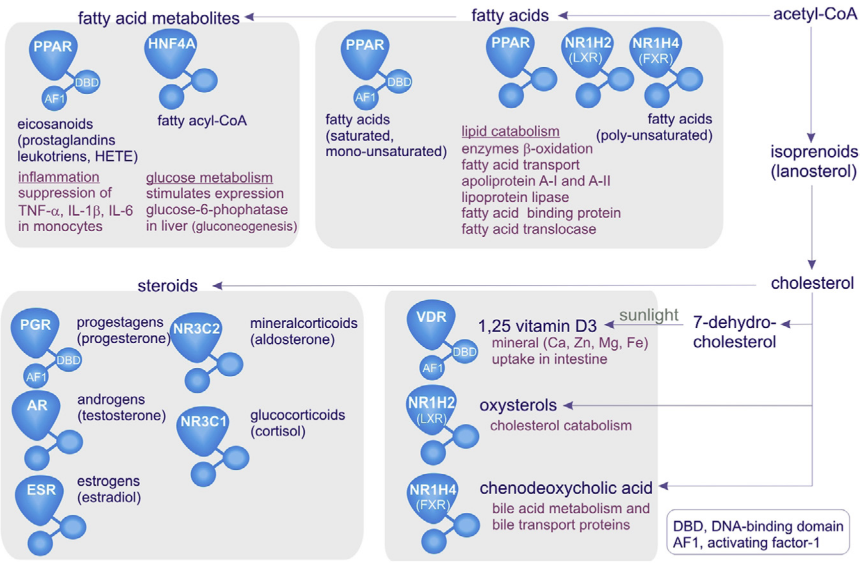


FIGURE 8-5 Control of lipid metabolism by nuclear receptors.

of phenotype, and the regulation of metabolic processes, such as cholesterol, bile acid, and fatty-acid metabolism. With respect to lipid metabolism, nuclear receptors are part of a tight network that controls expression of enzymes and transporters (Figure 8-5). Disorders of these systems can lead to infertility, obesity, diabetes, and cancer. The receptors act principally by binding DNA and controlling transcription.

PEROXISOME AND PEROXISOME PROLIFERATION

The peroxisome was first described in 1954 in the thesis of a Swedish student J. Rhodin (Karolinski Intitule, Stockholm, Sweden) as a small single membrane organelle (microsome) present in the proximal tubule cells of the mouse kidney. Further exploration and identification was carried out by the team of Christian de Duve, University of Louvain, Belgium, who in 1967 separated peroxisomes from other components (in particular mitochondria and lysosomes) by density-gradient centrifugation (de Duve, 1969). A major function of the peroxisome is the conversion of long-chain fatty acids into medium chains which, apparently, are more amenable to usage by mitochondria (in the process of production of ATP). They owe their name to the fact that they produce hydrogen peroxide (H_2O_2) which is released early in the process

continued

PEROXISOME AND PEROXISOME PROLIFERATION *(cont'd)*

of β-oxidation of fatty acids (in a reaction catalyzed by the enzyme Acyl CoA oxidase). Peroxisomes acquire their lipids and membrane proteins from the endoplasmic reticulum, whereas they import their matrix proteins directly from the cytosol (van der Zand and Tabak, 2013). In rodents, the number of peroxisomes increases (referred to as "peroxisome proliferation") when liver parenchymal cells are continuously exposed to hypolipidemic drugs (e.g., clofibrate), plasticizers (e.g., di-(2-ethylhexyl)-phthalate), or herbicides (e.g., 2,4,5-tricholophenoxyacetic acid). This proliferation is associated with an increased expression of genes encoding fatty acid β- and ω-oxidation enzymes and may eventually result in the cell transformation (cancer) (Reddy and Rao, 1986). Proteins that bind the above mentioned molecules were named peroxisome proliferator-activated receptors (PPAR) and it was shown that they directly induce the expression of Acyl CoA oxidase (Issemann and Green, 1990; Dreyer et al., 1992; Tyagi et al., 2011). Peroxisome proliferation is less obvious in humans but PPARs do nevertheless regulate expression of genes involved in lipid metabolism (Grygiel-Gorniak, 2014).

XENOBIOTIC RECEPTORS

NR1I2 (PXR, pregnane X receptor) and NR1I3 (CAR, constitutive androstane receptor) are expressed in the liver and gut. They coordinate protection against potentially toxic compounds by enhancing the expression of drug transporters and drug-metabolizing enzymes of the cytochrome P450 family. The receptors are said to be constitutive active but xenobiotics can further increase their activity. They form dimers with XRX. NR1I2 (PXR) is stimulated by a large variety of pregnenolone derivatives (natural precursor for all steroid hormones), among which is 5β-pregnane-3,20-dione. It has a particularly large ligand-binding cavity and demonstrates low-affinity binding for a wide range of ligands, such as antibiotics, antimycotics, and bile acids. NR1I3 (CAR) has its name because it can be inhibited by superphysiological concentrations of the testosterone metabolites androstanol and androstenol (collectively named androstanes) (Kliewer et al., 1998; Moore et al., 2000).

FXR, PXR, AND CAR IN BILE-ACID METABOLISM AND ELIMINATION

Bile acids are hepatotoxic and when they accumulate in the liver cell they occupy the FXR (NR1H4), and to a much lesser extent the receptors PXR (NR1I2) and CAR (NR1I3). In mice these nuclear receptors will then repress enzymes involved in the production of bile acids, for instance cholesterol 7-α-hydroxylase (CYP7A1) which is rate limiting in bile-acid synthesis, and activate expression of enzymes that process the metabolites for export, such as CYP3A3, CYP2B10, SULT2A1, and UGT1A1. They will also increase expression of alternative bile-acid transporters such as MPR3 and -4. Collectively, these changes relieve the liver from their toxic load (Traumer et al., 2005). Accumulation of bile acids, cholestasis, can be caused by obstruction of the ducts that lead to the gall bladder (parasites, gallstones, or cancer) and through liver diseases caused by, for instance, hepatitis viruses, excess alcohol, or cancer.

CYTOCHROME P450 PROTEINS

These heme proteins are oxidoreductases that catalyze the oxidation (loss of electron through transfer) of many endogenous and exogenous hydrophobic compounds, such as fatty acids, sterols, prostanglandins, leukotrienes, and a wide range of xenobiotics. Some 40 different P450 protein families have been identified on the basis of their sequences. Humans express 57 different P450 proteins which are classified with the prefix CYP. CYP3A4 is the predominant P450 and it is prominently expressed in adult human liver. It is thought to be responsible for the metabolism of some 60% of all clinically used drugs. For more information we refer to OMIM entry 124,010.

In evolution, the nuclear receptors first appeared in metazoans (animals and sponges) but are absent from plants, algae, fungi, and protists (but see text box (Phelps et al., 2006)). A retinoic-acid receptor, remarkably homologous with the vertebrate retinoid X receptor (RXR), is present in Cnidarians (animals with mostly radial symmetry like jellyfish, hydras, and corals) (Kostrouch et al., 1998). In the worm *Caenorhabditis elegans* there are 270 nuclear receptors, exceeding by far the 49 in human (Robinson-Rechavi and Laudet, 2001). However, as with other invertebrates, this species lacks receptors for adrenal steroids (aldosterone and cortisol) and sex steroids (estradiol, progesterone, and testosterone). In vertebrates, these hormones regulate reproduction, differentiation, development, and homeostasis. It is likely that receptors for steroid hormones first arose in a cephalochordate such as *amphioxus* (Baker, 2003).

Maybe not quite as absent from fungi as was formally thought. A combination of sequence profile searching, with structural predictions at a genomic scale, revealed heterodimeric transcription factors in yeast that contain ligand-binding domains that resemble those of animal receptors (Phelps et al., 2006). Although they share little sequence homology, the resemblance of the ligand-binding structural folds in yeast Oaf1 and Pip2 and the vertebrate PPAR–RXR heterodimers is evident. The ligand-binding domains can bind the fatty acids such as oleate, which provoke heterodimer formation (though these may not be their preferred ligands).

Web-based resource

Hugo Gene Nomenclature Committee, nuclear hormone receptors family http://www.genenames.org/genefamilies/NR.

Receptor isoforms

Different mRNA splice variants and alternative sites of initiation of translation have been reported for nuclear receptors. The glucocorticoid receptor is one of the receptors studied most extensively and UniProt (entry P04150) reports 10 isoforms. Of these, the beta-isoform lacks a short stretch in the C-terminal sequence and as a consequence fails to bind hormone. It is widely expressed at low levels and has an intrinsic gene-regulatory function, but it may also contribute to glucocorticoid signaling. Glucocorticoid hormone makes cancer cells more susceptible to apoptosis, a phenomenon exploited in hormone therapy of hematological malignancies, but it may lose its therapeutic impact if cells express too many beta-isoforms. The isoform beta is more abundantly expressed in transformed cells and may be the reason of occasional resistance to glucocorticoid treatment (Greenstein et al., 2002).

Orphan receptors

Nineteen human nuclear receptors are currently designated as “orphans,” having no recognized ligands (not yet). In invertebrates, the proportion of orphans is higher still. Indeed, the receptors that have known ligands are only expressed in those subfamilies that evolved comparatively recently suggesting again that their ancestral forms were insensitive to ligands (Escriva et al., 2003). Orphan receptors that do not bind ligands may still be able to influence transcription. For example, NURR1(type 4) lacks a binding pocket due to the presence of several bulky hydrophobic residues but even so, it is a potent transcriptional activator and plays a role in the development of dopaminergic neurons in the brain (Wang et al., 2003). Loss of function is linked to Parkinson's disease and schizophrenia (Saucedo-Cardenas et al., 1998; Le et al., 2002). On the contrary, the orphan receptor reverse ErbA (type 1) functions as a transcriptional suppressor. Although orphan receptors appear not to be regulated by ligand, they can still be subject to regulation by other means, either they can team up with other transcription factors or they are subject to post-translational modifications such as phosphorylation/dephosphorylation or acetylation. We will return to examples later in the chapter. Some receptors have ligands permanently bound within the cavity and thus are unable to influence activity. In this respect, HNF4A (type 2) has a constitutively bound fatty acid and the overall molecular conformation corresponds to a transcriptionally active receptor (Wisely et al., 2002).

DOMAIN ARCHITECTURE AND GENERAL STRUCTURE OF THE DNA–PROTEIN COMPLEX

The general structure of the nuclear receptors is highly conserved, consisting of a set of common functional domains (Figure 8-6(a)). There is a highly variable N-terminal domain (A/B), a highly conserved DNA-binding domain (DBD) with twinned zinc-finger motifs, a D domain and a C-terminal segment (E/F) that contains the ligand-binding domain (LBD). There are two activation function sites, AF-1 and AF-2. AF-1 is in the N-terminal domain and is ligand independent, while AF-2 is located in the LBD and is ligand dependent (if there is a ligand). There are also sites that determine nuclear localization and dimerization. Most of the nuclear receptors form dimers, homo- or heterodimers, with the stereogenic receptor-1 (NR5A1) being a known exception (return to Figure 8-4). The relative positioning of the different domains have been studied in more detail for RXR-containing heterodimers (Rochel et al., 2011; Orlov et al., 2012). The LBD and DBD are separated by a hinge region. The LBD are positioned at the 5′-end of the DNA-response element and the complex forms an asymmetric dimer. The rigid extended hinge region (CTE) of the VDR (or RAR) receptor positions the DBD at the downstream position, the 3′end of the DNA-response element (Figure 8-6(b)). Note that the modulating region (AF-1) of the VDR wraps around the DNA and may play a role in determining binding efficacy.

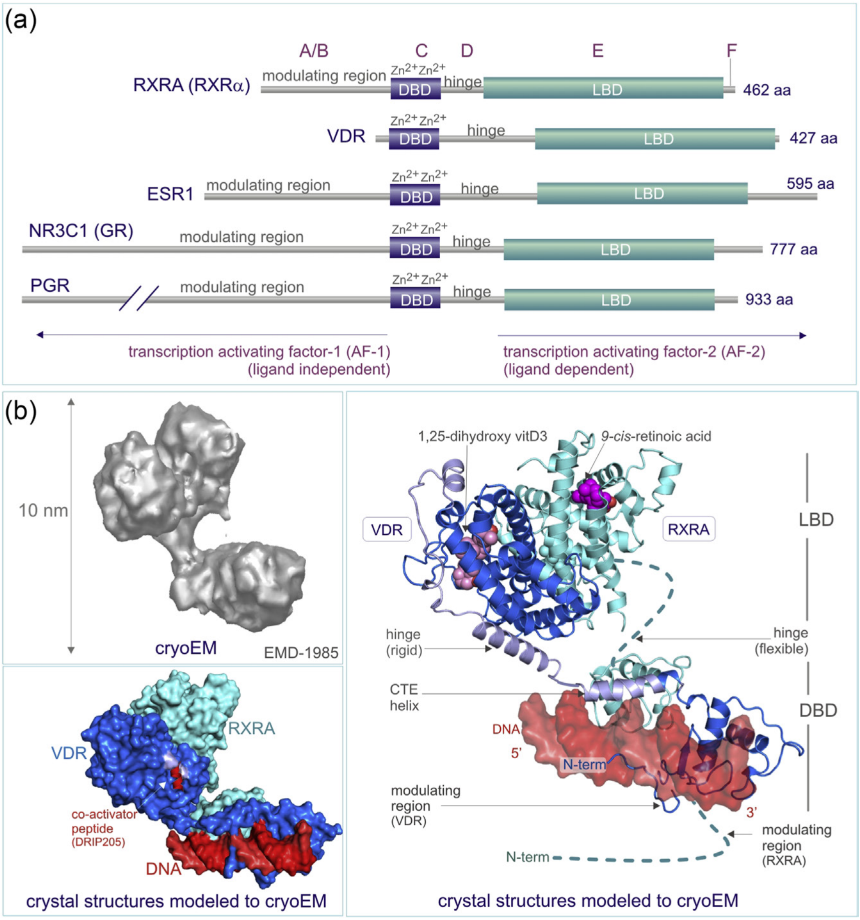


FIGURE 8-6 **Domain architecture and molecular structure of nuclear receptors.** (a) Domain architecture. The DNA-binding domain and ligand-binding domain are conserved, whereas the modulating and hinge regions are much more variable. The nuclear receptors have two segments that control gene transcription, named activating factor-1 (equivalent of A/B region or modulating region) and activating factor-2 (equivalent of the E and F regions). Abbreviations: DBD, DNA-binding domain; LBD, ligand-binding domain. (b) Overall structure of the VDR/RXRA receptors bound to DNA. Notice the asymmetric DNA binding and the 3′-positioning of the VDR DNA-binding domain relative to the one of RXRA. The rigid hinge (CTE helix) of VDR determines the position on the DNA, whereas the more flexible hinge of RXRA adapts its position depending on the distance of the second binding site (see also Figure 8-8). The cryo-electromicroscopy-derived three-dimensional structure was obtained from the EMBL-EBI EM-data Bank, entry number EMD-1985. Pdb coordinates of the DVR–RXRA dimer were kindly provided by Bruno Klaholz, Department of Integrated Structural Biology, IGBMC, Illkirch, France.

Molecular detail of the nuclear receptor

Many crystal structures have been determined of the LBD in complex with natural and synthetic ligands. These structures reveal a conserved core of 12 α-helices (H1–12) arranged into a three-layered sandwich fold (Huang et al., 2010). This arrangement generates a mostly hydrophobic cavity in the lower half of the domain. In all hormone- or agonist-bound structures, the ligand cavity is sealed by the C-terminal helix H12. This conformation is referred to as the active conformation because it favors the recruitment of co-activators. The ligand-binding pocket is surrounded by helices H3, H4/5, H7, and H11 and lined by nonpolar and aromatic residues (alanine, methionine, leucine, and phenylalanine). The two ends of the pocket contain polar residues such as arginine, glutamate, and histidine (see Figure 8-7(b) depicting the estrogen receptor bound to the agonist DES). The pocket varies in size among the different nuclear receptors. For estrogen (ESR), glucocoriticoid (NR3C1), mineral corticoid (NR3C2), progesterone (PGR), androgen (AR), vitamin D3 (VDR), all-*trans*-retinoic acid (RAR) and 9-*cis*-retinoic (RXR), the cavity is matched to ligand size. The corresponding receptors bind their ligands with high affinity (K_D in the nanomolar range). Then there is the group of receptors that bind with low affinity (micromolar range). These include oxysterol (NR1H2/3 or LXR) and bile-acid receptors (RN1H4/5 or FXR), which have been termed metabolic sensors, rather than receptors. They cope with a wider variety of ligands. The xenobiotic receptors (NR1I2 or PXR and R1I3 or CAR) have one of the largest and most flexible cavities, nearly twice that of the very high-affinity thyroid-hormone receptor. Conversely, the orphan receptor NR4A2 (NURR1) has virtually no cavity. Importantly, binding of ligand brings about a change in the length and the position of helices H11 and H12. This in turn creates binding sites for proteins that facilitate gene transcription (co-activators).

The DBD of nuclear receptors contain two Zn^{2+}-coordination sites composed of four cysteine residues and therefore referred to as C4-type zinc fingers. The zinc fingers nucleate the protein fold. The DBD of steroid receptors contain two essential sequences, one that is involved in protein dimerization, constituting the D-box (PATNQ), and the other in DNA binding, the P-box (EGCKA). The helix formed by the P-box recognizes the 5′-AGGTCA-3′ sequence in the major groove of the DNA (Figure 8-7(c)). The non-steroid nuclear receptors naturally contain a DNA-binding P-box but lack a D-box. Interactions between the two DBD are much more diffuse.

The P-box of the other steroid receptors is composed of GSCKV and it recognizes 5′-AGAACA-3′, whereas the P-box for receptors that form heterodimers with RXR is composed of EGCKG. A mutation of the GR

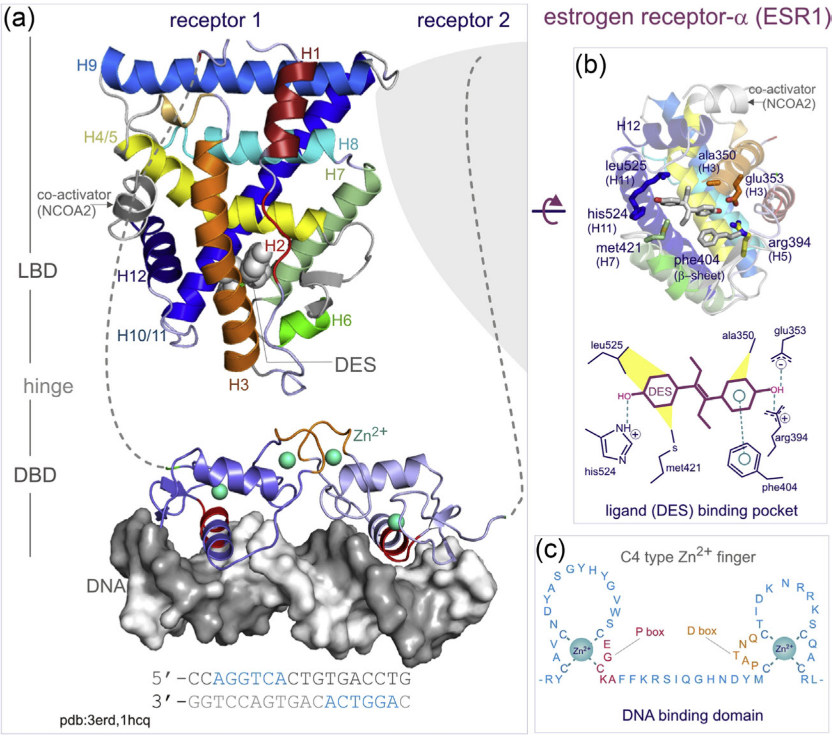


FIGURE 8-7 **Molecular detail of the estrogen receptor.** (a) Crystal structures the estrogen receptor (ESR1). The ligand-binding domain, on top, has a conserved core of 12 α-helices (H1–12) arranged into a three-layered sandwich fold. This arrangement generates a hydrophobic cavity in the lower section which is occupied by the estrogen agonist DES (in light gray). The helices are colored ranging from red (H1) to dark blue (H12). Helices H4 and H12 are involved in binding of activators or suppressors of gene transcription (a short stretch of the co-activator NCOA2 is shown here in light gray). A hinge region connects the ligand binding with the DNA-binding domain. The image shows a dimerized DNA-binding domain (receptors 1 and 2) which are held together by both protein–protein (orange loops) and protein–DNA interactions (red helices). The zinc finger motives are characteristic of nuclear receptors. The estrogen receptor recognizes a palindromic sequence 5′-AGGTCA-3′ (inverted repeat) and as a consequence the DNA-binding domains bind head to head. (b) Details of the ligand-binding pocket of ESR1. The pocket is lined by hydrophobic residues with (at the bottom and the top) one or two polar residues. Ligand binding affects the position of helices 11 and 12. The binding of the receptor agonist DES leads to the recruitment of the co-activator NCOA2. (c) Schematic representation of the two zinc-finger motifs of the DNA-binding domain. Note the coordination of Zn^{2+} by four cysteines (C4 type). The P-box sequence EGCKA binds the 5′-AGGTCA-3′ DNA sequence, whereas the D-box sequence PATNQ is involved in protein–protein interaction.

P-box, G→E, produces a receptor that can bind either GR or ER response elements. Complete swapping of the GR P-box for that of ER, confers an ability to bind and activate at estrogen response elements, eliminating glucocorticoid responsiveness.

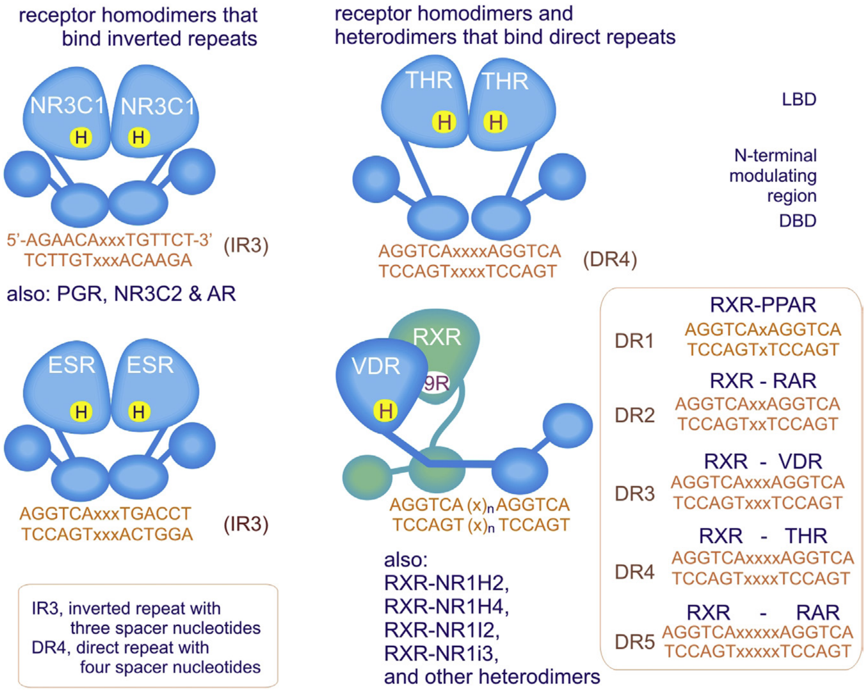


FIGURE 8-8 **Dimerization of nuclear receptors and binding to DNA half-sites.** Steroid hormones bind as homodimers to inverted repeats separated by a spacer of three base pairs (IR3). The example shows the glucocorticoid and estrogen receptor. Other nuclear receptors bind to direct repeats but separated by different spacer lengths. The thyroid receptor (THR) binds as a homodimer to a direct repeat separated by four base pairs (DR4) but it can also bind to the same sequence in a dimer with RXR. PPAR, RAR, and VDR all bind to DNA in association with RXR. The specific is determined by the spacer length between the direct repeats. Thus RXR/PPAR binds DR1 sites whereas RXR/RAR binds DR2 sites. H, hormone, 9R, 9-*cis*-retinoic acid, IR, inverted repeat, DR, direct repeat.

DNA response elements of nuclear receptors

The DNA-binding sites, response elements, consist of two half-sites, each of six base pairs. We distinguish two main types: binding of homodimers to inverted repeats, such as the steroid-hormone receptors, and binding of homo- or heterodimers (with RXR) to direct-repeat sites, such as the thyroid-hormone receptor, the retinoic-acid receptor, the vitamin D3 receptor, and peroxisome proliferation-activating receptor (Figure 8-8). All half-sites are separated by a number of base pairs. For the two inverted-repeat sequences the spacer number is always three (termed IR3), whereas for the direct repeats the number of spacers varies from 1 to 5 (termed DR1–DR5). Because the DNA sequence of the direct repeat is similar for all nuclear receptors concerned, a measure of discrimination is provided by varying the spacer length. Each additional base pair separates the half-sites by 3.4 Å and introduces a relative rotation of 36°. The thyroid hormone-receptor homodimer (THR–THR) or heterodimer (RXR–THR) recognizes DR4. The RXR–PPAR dimer preferably recognizes DR1, the RXR–RAR recognizes DR2 or DR5, and the dimer RXR–VDR preferably recognizes DR3. Note that the DBD of RXR is always situated upstream (5′) of its partner. Further specificity may come from binding of the modulating region to upstream or downstream DNA sequences (return to Figure 8-6(b)). It is noteworthy that nuclear-receptor binding sites are found in typical promoter regions, upstream and nearby the transcription start site, but a significant number are located in intragenic regions at distances above 25,000 base pairs (25 kb). Extensive chromosome folding will be required in order to exert an influence on transcription.

LBD are molecular switches

The effect of ligand binding is a conformational change which is best visualized by changes in the length of helix H11 and the position of helix H12. We will discuss two examples, the retinoic acid receptor-α (RARA) and the estrogen receptor-β (ESR2). The retinoic acid receptor is a functional transcription factor both in its unliganded (aporeceptor) and liganded states. Without ligand it recruits the nuclear cofactor NCOR1 through recognition of a specific sequence named the repressor box (in NCOR1 the sequence is T**L**ADH**I**Q**II**TQD**F**, with conserved residues in bold). It was found however that certain ligands could enhance the repressor function of the receptor and one of them, BMS493, qualifies as inverse agonist. It stabilized the receptor in a configuration where helix H11 is nearly absent and instead forms a short β-sheet which binds in an antiparallel fashion to a short β-sheet of the repressor NCOR1. Under this condition helix H12 is not detectable in the crystal (Figure 8-9(a)). Addition of all-*trans*-retinoic acid, or the agonist AM580, causes a lengthening of H11 and attachment of H12 to the entry of the ligand pocket. In this conformation the receptor recognizes a different sequence, the activator box, and recruits the co-activator NCOA1 (sequence HKI**L**HR**LL**QE, with conserved residues in bold). The antagonist MBS614 shortens the H11 helix and places H12 precisely where the receptor would normally interact with effectors thus preventing access of either NCOR1 or NCOA1. Under all conditions, helix H4 too plays a role in the coordination of helix H12 and as a co-activator or a co-repressor (not shown) (le Maire et al., 2010).

The different structures of the estrogen receptor reveal the same conformational changes as observed for the retinoic-acid receptor. In their agonist-bound state, both receptors have a near identical overall structure with a difference that the estrogen receptor binds the co-activator NCOA2 (not NCOA1). With respect to antagonist-bound receptors, two different

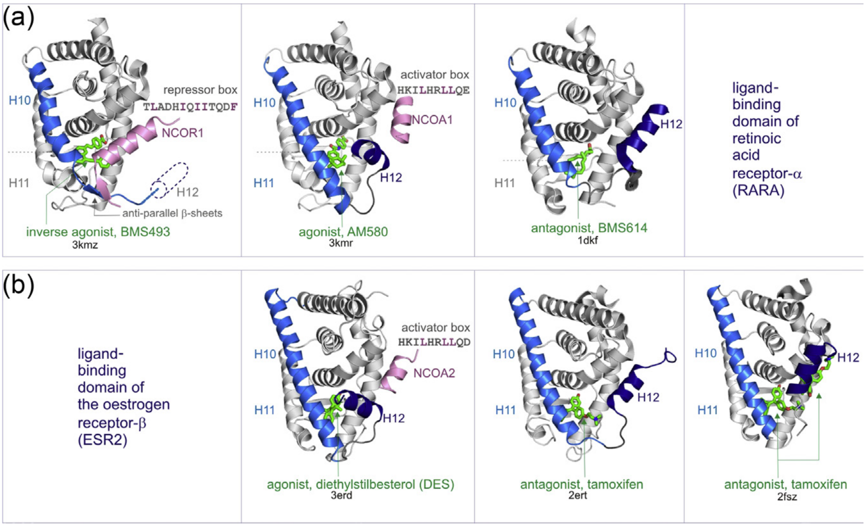


FIGURE 8-9 Ligand-mediated conformational changes of the nuclear receptor and recruitment of co-repressors or co-activators. (a) Crystal structures of the retinoic-acid receptor-α (RARA) bound to an inverse agonist, agonist, or antagonist. Note the changes in the composition of helix H11 (blue), the position of helix H12 (dark blue), and the attachment of NCOR1 (co-repressor), or NCOA1 (co-activator), or the lack of attachment of co-regulators in the case of the antagonist BMS614. (b) Crystal structure of the estrogen receptor bound to an agonist (DES) or antagonist (tamoxifen). Note the astonishing structural resemblance between the agonist-bound estrogen and retinoic-acid receptor in particular with respect to the positioning of helix H12. A particular feature of the estrogen receptor is that tamoxifen (antagonist) can bind two positions: inside the hydrophobic cavity and within the binding groove for the co-activator NCOA2. Crystal structures have been obtained with either one or two binding sites occupied. Antagonistic action is enhanced in the latter case. Pdb: 3kmz, 3kmr, 1dkf, 3erd, 2ert and 2fsz.

structures have been reported. In the first structure we observe a single antagonist which places helix H12 within the co-activator binding groove and prevent its access. In the second structure two tamoxifens bind to the receptor: one inside the ligand-binding pocket and one precisely within the co-activator-binding groove (the NCOA2-binding site) (Figure 8-9(b)). In the latter configuration inhibition of the gene transcription is even more effective (Wang et al., 2006).

Steroid hormone receptors bind exclusively as homodimers on response elements but the other receptors often form dimers with RXR. With respect to receptor heterodimers, the ligand-dependent activity of RXR is subordinated to that of the liganded partner. Binding of ligand to RXR causes a change in conformation but it fails to replace the repressor complex with an activator complex. Co-activators preferably bind to the liganded partner of RXR and only then does the activity shift from repression to activation of gene transcription (Germain et al., 2002; Rochel et al., 2011). PPAR/RXR heterodimers form an exception, they are "permissive" and each single ligand is sufficient to induce transactivation.

DNA as an allosteric mediator of nuclear receptors

The DNA sequences shown in Figure 8-3 are not fully conserved among species and may vary in one or more base pairs. The differences in DNA sequences do not necessarily affect binding of the nuclear receptor but they do affect the conformation of the DBD. For the glucocorticoid receptor (NR3C1) this, in turn, was shown to affect the efficacy of gene induction (using an artificial promoter coupled to the luciferase gene) because of increased or reduced recruitment of co-activators. In short, DNA acts as an allosteric regulator of nuclear receptors (Meijsing et al., 2009). The importance of DNA was also shown by the observation that certain promoter regions bind the glucocorticoid receptor but through a sequence that differs profoundly from the activating sequences shown above. This is the case for the thymic stromal lymphopoietin gene (TSLP), coding for a pro-inflammatory cytokine, which contains a glucocorticoid receptor response element with the sequence 5′-CGCCTCCGGGAGAGCT-3′. Cortisol, or one of its agonists, rather than activating the gene reduces mRNA levels by 50%. The sequence turns out to act as a negative response element (nGRE). It holds the DBD apart in a tail-to-tail orientation and rather than recruiting enzymes that promote gene transcription, the negative response-element brings about the formation of a nuclear-receptor repressor complex (Surjit et al., 2011; Hudson et al., 2013). The picture that emerges from these findings is that the precise conformation of both the LBD and the DBD play a role in the composition of the regulatory complex that develops around the nuclear receptor.

The genomic mode of action of nuclear receptors

Nuclear receptors can be divided into two groups, one representing receptors that shuttle between the cytoplasm and the nucleus, predominantly steroid receptors, and the other representing receptors that permanently reside in the nucleus (all the rest). In the first group, ligand binding is assisted by chaperone proteins of the heat-shock protein family, HSP70, and HSP90 (Rohl et al., 2013). This has been studied most extensively for the glucocorticoid receptor (NR3C1). Although the picture is far from complete, ligand binding seems to occur in two different configurations: when a single receptor is bound to HSP70 and HSP90 or when two receptors are bound to a complex of HSP90, P23 (co-chaperone), and FKBP4 (immunophilin). In the first case, two rounds of receptor binding must occur for the establishment of a ligand-loaded receptor dimer. The process requires multiple cycles of ATP hydrolysis (Figure 8-10) (Lorenz et al., 2014). It is not firmly established where exactly the nuclear-receptor dimer detaches from the chaperone complex. This may occur in the cytoplasm or in the nucleus. It has been shown that the immunophilin FKBP4 links the complex to dynein and this motor protein plays a role in the rapid (time-scale of 4–10 min) transport of the receptor to the nucleus (Pratt et al., 2004).

Following translocation, the receptor dimer binds one of the above described DNA-response elements. In the presence of a natural ligand or an agonist, the dimer recruits co-activators (NCOA's) and these in turn recruit (a) *N*-methyl-transferases, which methylate arginyl residues on a variety of proteins involved in DNA packaging and transcription regulation (example CARM1), (b) histone acyl-transferases (example of EP300 and CREBBP), and (c) a nucleosome remodeling complex that goes under the name SWI/SNF (involved in altering the position of nucleosomes along DNA) (Figure 8-11). The resulting chromatin remodeling makes the

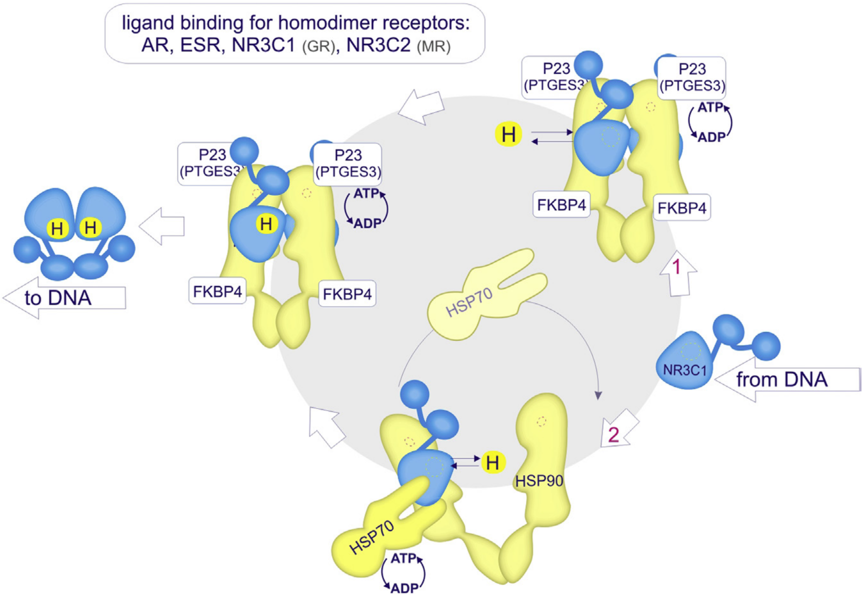
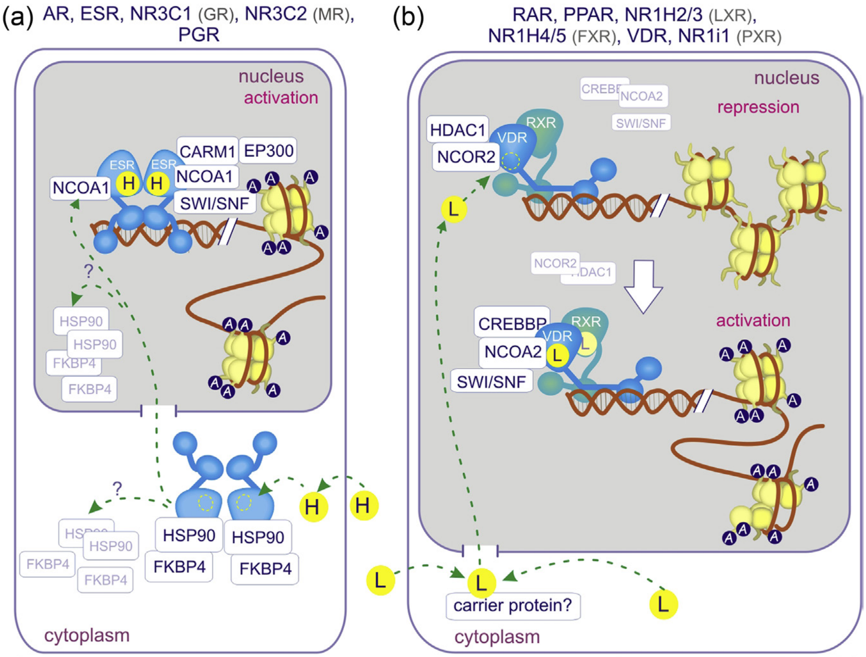


FIGURE 8-10 Detail of heat-shock proteins involved in loading the steroid receptors with their ligand. The chaperones of the heat-shock protein family assist in the loading of ligands for nuclear receptors in two fashions. (1) The unliganded receptor directly binds HSP90 in complex with P23 and FKBP4 or (2) the receptor binds HSP70 first and is then presented to HSP90. The second event is repeated twice so that a receptor dimer forms (embraced by HSP90). The ligand-loaded receptor dimer detaches and enters the nucleus. The process requires a number of ATP hydrolysis cycles. *Image based in part from information obtained from Lorenz et al. (2014).*



CREBBP (CBP), CREB-binding protein (histone acyltransferase)
EP300 (P300), E1A-associated protein 300 kDa (histone acyltransferase)
CARM1, co-activator-associated arginine methyltransferase
HDAC1, histone deacetylase-1
SWI/SNF, SWitch/Sucrose NonFermentable
NCOA1 (SRC-1), nuclear receptor co-activator 1
NCOA2 (SRC-2, TIF2), idem
NCOR2 (SMRT), nuclear receptor co-respressor 2

FIGURE 8-11 **Nuclear receptors in action.** (a) Steroid receptors are charged with ligand in the cytoplasm when complexed to chaperones of the heat-shock protein family (HSP90 and co-factors). After translocation into the nucleus, the liganded receptors bind specific DNA-response elements and recruit nuclear co-activators such as NCOA1. This in turn recruits enzymes that modify histones and transcription regulators through methylation and acetylation (CARM1 and EP300, respectively) and redistributes nucleosomes along the DNA with the help of the SWI/SNF complex (multiple proteins). The ensuing chromatin remodeling renders the DNA more accessible for gene transcription. We note that unliganded receptors of all types may remain in the nucleus and exert a repressor function. (b) Receptors that form hetero-dimeric complexes with RXR do not shuttle in and out of the nucleus of and when lacking a ligand they recruit the nuclear co-repressor NCOR2 (example of VDR/RXR) and the histone deacetylase HDAC1. As a consequence DNA is poorly accessible and gene transcription is prohibited. The situation changes with the arrival of a natural ligand (vitamin D3) or and agonist, and the receptor complex now recruits the nuclear co-activator NCOA2, which in turn facilitates the arrival of CREBBP, a histone acyltransferase. The subsequent loosening of the chromatin leads to enhanced gene transcription. How the ligand reaches its receptor is not clear to us but normally it requires a carrier protein. H, hormone (estrogen), L, ligand (vitamin D3, all-*trans*-retinoic acid, or 9-*cis*-retinoic acid).

DNA more accessible for gene transcription. The loss of ligand does not necessarily cause immediate detachment from DNA. It has been shown for the unliganded estrogen receptor-α (ESR1) that the dimer remains attached and, although transcriptionally unproductive, holds the gene promoter in a ready-to-go state (“poised state”) (Metivier et al., 2004).

SWI/SNF is derived from SWItch/Sucrose NonFermentable, a protein complex found in both eukaryotes and prokaryotes. The human analogs of bacterial SWI/SNF genes are BAF (SWI/SNF-A) and PBAF (SWI/SNF-B). BAF in turn stands for "BRG1-or HRBM-associated factors," and PBAF is for "polybromo-associated BAF." BRG1 (SMARCA4) and HRBM (SMARCA2) are ATP-dependent helicases. Loss-of-function mutations are detected in human cancers suggesting that the complex may qualify as a tumor suppressor.

With respect to the other receptors (RAR, PPAR, etc.), they are already in the nucleus and bound to DNA where they act as transcription repressors. For VDR/RXR it was shown that the complex recruits NCOR2 which in turn attracts the histone deacetylase HCAC1. Deacetylation of histones renders the DNA less accessible for gene transcription because it makes the nucleosome/DNA interaction more rigid. Ligands for these receptors are intracellular metabolites, hormones, or xenobiotic substances that have entered the cell. It is unclear to us how these substances enter the nucleus; carrier proteins seem obligatory. Binding of ligand causes a conformational change in the nuclear receptor as depicted in Figure 8-9 and nuclear co-repressors are replaced by co-activators which in turn recruit chromatin remodeling enzymes such as histone deacetylases (HDAC) and the nucleosome remodeling complex SWI/SNF. The process of exchange of co-regulators involves the recruitment of the ubiquitylation machinery and necessitates proteasome-dependent degradation of NCOR1 or -2 (Perissi and Rosenfeld, 2005).

The transcription complex is more complex than depicted in Figure 8-11, numerous other factors associate indirectly with the nuclear receptor as well as with transcription factors bound to other response elements of the same gene. Moreover, the regulatory elements assemble around the basal transcription machinery, called the core promoter, which attaches to DNA just upstream of the coding sequence (often recognized by a TATA sequence). The ensemble, regulatory plus core elements, creates discrete nuclear sub-compartments also known as "transcription factories" (Osborne, 2004). For a detailed listing of nuclear receptor co-regulators we refer to references (Hernanson et al., 2002; Cosma, 2002).

NUCLEAR RECEPTORS IN CONTEXT: CROSS-TALK WITH OTHER TRANSCRIPTION FACTORS

Transactivation

Nuclear receptors, like any other transcription factor, do not drive gene expression in isolation; they are always part of a complex of transcription factors bound to a composite response element (sometimes referred to as an “enhanceosome” (Kassel and Herrlich, 2007; Ratman et al., 2013)). Moreover, possessing a nuclear receptor response element is not necessarily a save measure for predicting transcriptional control. Not all promoter regions are necessarily accessible to transcription factors. When comparing binding of glucocorticoid receptor to DNA in two different cell lines, one derived from the mammary gland and the other from the pituitary, it was revealed that roughly 80% of the protein binds to “accessible” chromatin but that the two cell lines only share 11% of the glucocorticoid receptor-binding sites. What this means is that each cell line has different accessible sections in their genome and this explains, in part, cell-specific hormone action (John et al., 2011). The glucocorticoid receptor is found in promoters which also carry proximate response elements for the transcription factors CREB, C/EBP, Oct-1 (POU2F1), AP-1 (JUN-JUN or FOS-JUN), or STAT1-3. The transcriptional outcome of “cross-talk” between the glucocorticoid receptor and these transcription factors is mainly positive (with the exception of C/EBP and FOS-JUN) (Figure 8-12). The efficiency of fully occupied composite response elements relies on cooperativity with respect to binding to DNA, meaning that the binding of one transcription factor facilitates the binding of another, and with respect to the recruitment of enzymes that modify the chromatin (CREBBP for instance is a multidomain protein which can interact with multiple proteins) (Vo and Goodman, 2001) (return to Chapter 7, “Bringing the Signal into the Nucleus: Regulation of Gene Expression”).

In certain promoters the response elements do overlap and the action of transcription factors is necessarily competitive, the one that gets access to the DNA is the winner, rather than cooperative.

PDB-101 “structural view of biology” entry for a composite response element (the “enhanceosome”). http://www.rcsb.org/pdb/101/motm.do?momID=122.

Transrepression

Numerous genes lacking nuclear-receptor response element in their promoter are nevertheless sensitive to steroid hormones, in particular to cortisol (glucocorticoid) (Ogawa et al., 2005). It was shown that nuclear receptors can also influence gene transcription by binding to other transcription factors (tethering) rather than to DNA. Binding partners are manifold, such as JUN, IRF3, NFκBp65 (RELA), SMAD3/4, STAT5/6, and T-bet (TBX21). The

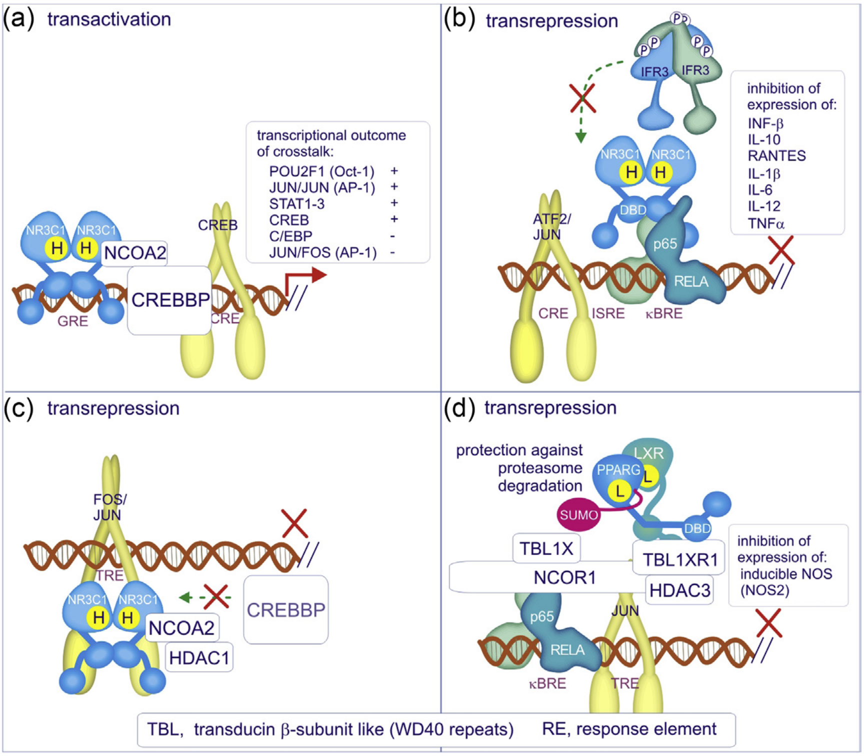


FIGURE 8-12 **Nuclear receptors in context.** (a) When promoter regions contain a nuclear-receptor response element, for instance the glucocorticoid-response element GRE, receptor occupation often results in transactivation. The nuclear receptor (NR3C1) enhances the action of other transcription factors by a cooperative mechanism that involves DNA binding and the recruitment of chromatin-modifying enzymes (CREBBP for instance). Exceptions are the cross-talk between NR3C1 and C/EBP or JUN/FOS (AP-1 complex). (b) In the absence of nuclear-receptor response elements, cross-talk can occur through protein–protein binding. In the presence of cortisol, the glucocorticoid receptor (NR3C1) binds NFkBp65 (RELA) and this prevents attachment of IFR3. Expression of inflammatory cytokines is hold-in-check (important role of cortisol). (c) The glucocorticoid receptor can also bind to an FOS/JUN complex and although it binds a co-activator of transcription in the presence of cortisol (NCOA2), this protein fails to recruit CREBBP and instead interacts with deacetylation enzymes (HDAC1), thus preventing gene transcription. (d) The heterodimer complex PPARG/LXR also can regulate gene transcription through protein–protein interaction. In the example shown here, ligand-occupied receptors attach to TBL1XR1, an adaptor protein that couples NCOR1 (co-repressor) to HDAC3 (deacetylase). Normally, upon inflammation for instance, the repressor complex is destroyed by the proteasome, allowing co-activators to bind to the modified transcription factors. In this example it will lead to expression of inducible nitric oxide synthases iNOS (NOS2) and causing vasodilatation. Nuclear receptors prevent all this by avoiding the attachment of the ubiquitylation machinery to TBL1X (bound to NCOR1). Efficient transrepression requires SUMOylation of PPARG. *Information obtained from Glass and Saijo (2010).*

protein–protein interaction occurs through the DBD of dimerized nuclear receptors, although other domains are not yet excluded (work in progress) (Tao et al., 2001). These protein–protein interactions generally result in repression of transcription and the phenomenon is referred to as transrepression. Besides the glucocorticoid receptor, this mode of repression of gene transcription has been revealed for PPAR-γ (PPARG) and for NURR1 (Jiang et al., 1998; Glass and Saijo, 2010).

Multiple mechanisms exist but we highlight only three examples of transrepression (Figure 8-12). In the first, binding of the glucocorticoid receptor to NFκBp65 (RELA) prevents cooperative binding with the transcription factor IFR3. When the ligand-bound glucocorticoid receptor binds to JUN, in a complex with FOS, it turns its co-activator NCOA2 (GRIP1) into a co-repressor (Rogatsky et al., 2002). In the third mode of operation, demonstrated for PPAR-γ and LXR (NR1H2, -3), the ligand-bound nuclear receptors (occupied by fatty acids/prostaglandin D and oxysterols, respectively) prevent degradation of a repressor complex attached to an AP-1 complex (composed of JUN–JUN). As a consequence the complex cannot be exchanged with a co-activator complex and repression persists (Ghisletti et al., 2007). For this mode of operation, the nuclear receptor has to be modified by a short peptide which resembles ubiquitin and is named SUMO (small ubiquitin-like modifier). SUMOylation occurs in the LBD on a lysine in helix H7 (light green in Figure 8-7). Whether or not transrepresion requires a receptor dimer remains to be established. Of note, mice with dimerization-deficient glucocorticoid receptors are highly susceptible to inflammatory stimuli because they fail to “transrepress” the inflammatory response (Kleiman et al., 2012).

ADVERSE EFFECTS OF GLUCOCORTICOID AGONISTS

In order to suppress chronic inflammation, as occurs in asthma or rheumatoid arthritis, glucocorticoid agonists such as dexamethasone, prednisone, prednisolone, or fludrocortisone are applied systemically. Long-term treatment brings about a number of symptoms, collectively named Cushing syndrome (symptoms described by Harvey Cushing for patients with pituitary tumors). Among the symptoms we list diabetes (insulin resistance), osteoporosis (inhibition of osteoblast and enhancement of osteoclast activity), growth retardation and muscle wasting (inhibition of the mTOR pathway, increased expression of FOXO, and enhanced ubiquitin-proteasome activity), skin thinning (less collagen), increased fat deposition (moon face and fat belly), infertility in women (due to increased androgens), high blood pressure and mental disturbances such as depression, mania, anxiety, and cognitive dysfunction.

Nuclear receptors in context: cross-talk through receptor phosphorylation

Nuclear receptors contain many consensus phosphorylation sites and are therefore the target of multiple kinases. We distinguish four distinct consequences of phosphorylation; certain phosphosites facilitate receptor dimerization and ligand binding, others facilitate interaction with the RNA polymerase-II pre-initiation complex and yet others facilitate attachment of co-activators (NCOA's). Finally, a number of phosphosites cause the recruitment of ubiquitin E3 ligases, and these signal the destruction of nuclear-receptor complexes by the proteasome (Figure 8-13) (Rochette-Egly, 2003). Phosphorylation in the AF-1 segment is often caused by CDK7, a protein kinase attached to transcription factor II-H. TFIIH is one of the several general transcription-factor complexes that make up the RNA-polymerase pre-initiation complex. Phosphorylation of nuclear receptors by CDK7 increases the attachment with the pre-initiation complex and this in turn strongly promotes the initiation of gene transcription. Cell cycle protein kinases, such as CDK2, -4, and -6 (in complex with their cyclins), also phosphorylate sites in the AF-1 segment of a number of nuclear receptors and this may play a role in cell cycle-dependent gene transcription and in the regulation of cell differentiation (arrest of cell cycle).

Numerous signaling pathways emanating from growth factor receptors and hormones lead to the activation of protein kinases, such as JNK (MAPK8), JNK3 (MAPK9), p38 (MAPK14), ERK1 (MAPK3), PKC-δ (PRKCD), PKA (PRKACA), and PKB-α (AKT1). Many of these can phosphorylate the AF-1 segment and some the LBD. We illustrate the importance of phosphorylation with the surprising finding of 1991, that genital-tract formation could be restored in ovariectomized mice, thus lacking estrogen production, when epidermal growth factor (EGF) was administered via slow-release pellets. Moreover, the EGF treatment also caused the induction of expression of uterine lactoferrin, a major estrogen-inducible target (Nelson et al., 1991). Further studies revealed that EGF causes estrogen-receptor translocation to the nucleus. All this required the AF-1 segment of the receptor whereas the LBD-associated AF-2 was dispensable. This is why the AF-1 segment is referred to as the "ligand-independent" activating factor. The mouse estrogen receptor-α (ESR1) is phosphorylated by ERK1 (MAPK3) at serine-118 and indeed this facilitates the recruitment of co-activators (Bunone et al., 1996). Phosphorylation by the same protein kinase but at S294 (in the hinge region) facilitates the recruitment of E3 ligases which, through ubiquitylation and subsequent degradation by the proteasome, serve to terminate the response (Zhou and Slingerland, 2014). For ubiquitylation enzymes we refer

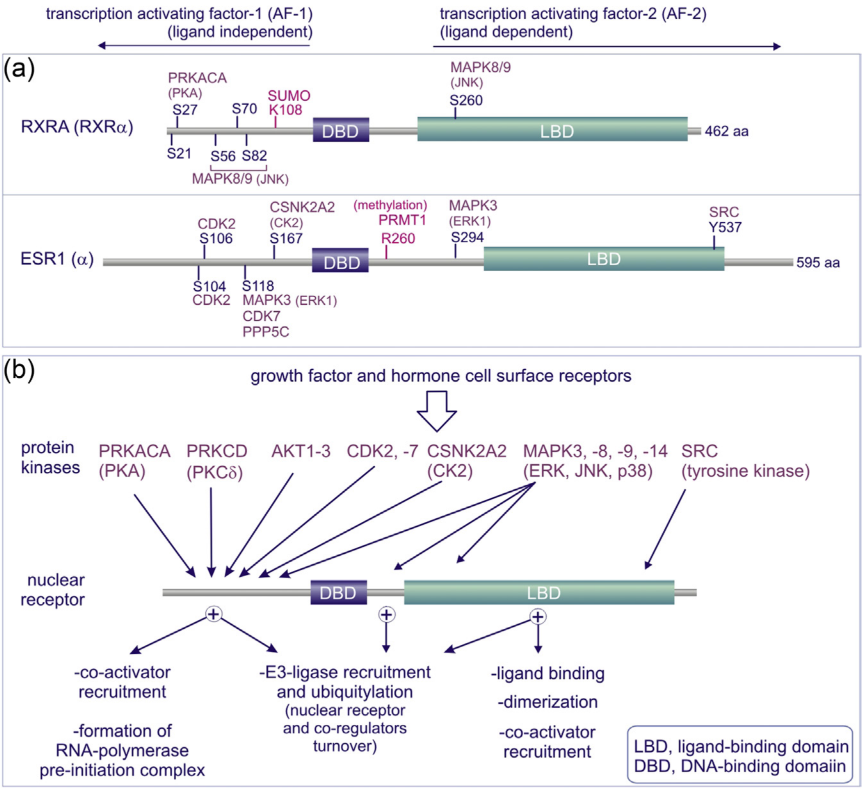


FIGURE 8-13 Phosphorylation sites and functional consequences of nuclear receptor phosphorylation. (a) Phospho-residues detected in the human RXRA and ESR1 receptors with their cognate protein kinases (or in the case of PPP5C, its phosphatase). Note the large number of phosphorylation sites in the AF-1 segment. Besides phosphorylation we also provide the example of a SUMOylation site on RXRA (on lysine-108) and a methylation site on ESR1 (on an arginine-260). The post-translational modifications shown are obtained from UniProt. Other modification sites certainly exist. (b) Numerous growth factor and hormone receptors contribute to the regulation of nuclear receptors. Phosphorylation by a large range of protein kinases generally facilitates nuclear-receptor transactivation. This occurs either through enhanced recruitment of co-activators or through a positive influence on the formation of the RNA-polymerase pre-initiation complex (transcription process). Phosphorylation also plays a role in signal termination as certain phosphosites recruit E3 ligases, enzymes that catalyze ubiquitin transfer onto protein and thus signal their destruction by the proteasome. *Information obtained from Rochette-Egly (2003).*

to Chapter 13, “Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signalling through Ubiquitinylation” (Figure 13-14). These studies brought to light the intricate relationship between cell membrane-mediated and nuclear receptor-mediated events. We will show below that nuclear receptors by themselves induce non-genomic signaling events.

NUCLEAR RECEPTOR TURNOVER AND CO-REGULATOR CYCLES

While studying recruitment of the estrogen receptor to DNA over time (3-h period), it was revealed that it binds the promoter region of the TFF1 gene (pS2 or breast-cancer estrogen-inducible protein) in a cyclic fashion: a repetition of binding transients that last for roughly 30 min. Co-regulators and RNA-polymerase-II follow the same up- and down-cycles but with a slight delay. Ubiquitylation and degradation of nuclear receptors and their co-regulators play an important role in these cycles. It is speculated that nuclear receptor clearance allows a more sensitive verification of the "hormonal state" of the cell and prevents overshoot in hormonal responses (Perissi and Rosenfeld, 2005).

NON-GENOMIC SIGNALING MODES OF NUCLEAR RECEPTORS

It soon became apparent that steroids employ mechanisms other than direct gene regulation. Responses to steroids were recorded in cells that lacked a nucleus (erythrocytes and platelets) or had an inaccessible nucleus (spermatozoa), they were recorded in a short time frame (in the seconds range), they occurred despite inhibition of transcription by actinomycin D and finally, cells were responsive to steroid conjugates that were unable to cross the plasma membrane (Lösel and Wehling, 2003). Non-genomic responses that paved the way to further studies are the rapid anesthetic effects of steroids in rats (Selye, 1942), the immediate reduction in vascular resistance (within 3 min) upon intravenous application of aldosterone in humans (Klein and Henk, 1963), and a rapid inhibition of Na^+ uptake by aldosterone in isolated erythrocytes from dogs that had their adrenal glands removed (Spach and Streeten, 1964). It is now clear that steroid hormones can bind cell membrane proteins and that they can induce immediate signaling events that we have described for G protein-coupled receptors (adrenaline and muscarinic acetylcholine receptors) and for growth factor receptors (further discussed in Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases"). Unfortunately, in many instances the detailed characterization of nuclear-receptor signaling complexes is still lacking, as is the identity of alternative ligand-binding sites (non-nuclear receptors). As an example, the presence of estrogen-binding sites at the plasma membrane was first reported in 1977 (Pietras and Szego, 1977) but until today it is not clear whether this is a truly novel membrane protein or a classic nuclear receptor, or a combination of the two.

It was shown that steroids, vitamin D3, and thyroid hormones activate heterotrimeric GTP-binding proteins such as Gαs and Gαq, they cause the production of cAMP (Aronica et al., 1994), progesterone raises intracellular free Ca^{2+} through its effect on voltage-gated Ca^{2+} channels (Lishko et al., 2011) or through activation of phospholipase Cβ (Le Mellay and Liberherr, 2000), alfaxalone (a neurosteroid) increases the γ-amino-butyric acid-induced chloride conductivity of GABAA (which explains the immediate anesthetic effect of steroids because the neurons become less excitable) (Cottrell et al., 1987; Paul and Purdy, 1992), aldosterone increases activity of the H$^+$/Na$^+$ exchanger (which explains the immediate effects of aldosterone on Na$^+$ uptake) (Wehling, 1995), and thyroid hormones stimulate the RAS–MEK–ERK and the PI3-kinase pathway through activation of the cytosolic tyrosine protein kinase SRC, through activation of the adhesion molecule integrin αvβ3 or through transactivation of the EGFR (Lösel and Wehling, 2003; Bergh et al., 2005; Stenzel et al., 2014). Three non-genomic effects are highlighted in Figure 8-14.

The estrogen and progesterone nuclear receptors each contain a proline-rich sequence (PPPLPPR) in their long N-terminal segment (in the AF-1 region). Proline-rich sequences are known to interact with the SH3 domain of members of the tyrosine protein kinase SRC (Boonyaratanakornkit et al., 2001; Ballare et al., 2003). The SH3 domain plays an essential role in the regulation of SRC activity as it normally wraps around the kinase domain and prevents activation (an inactive state referred to as the “latched conformation”) (return to Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases”). Binding to a proline-rich region of another protein relieves the inhibitory constraint and augments the chance of auto-phosphorylation and subsequent activation. Once active, SRC phosphorylates the adaptor protein SHC1 and this initiates a genuine growth factor pathway; starting with the recruitment of a nucleotide exchange protein at the membrane, activation of the monomeric GTPase RAS followed by a cascade of kinases that cause activation of the ERK1 (MAPK3). This signal by itself enhances cell proliferation but it also positively feeds back to the estrogen receptor and enhances its genomic effect (Razandi et al., 2004; Pedram et al., 2006). This non-genomic pathway causes *Xenopus* oocyte maturation, a process where, upon exposure to progesterone, the oocyte escapes from cell cycle G2-arrest and enters into first and second meiosis followed by fertilization.

THREE PRECISE DESCRIPTIONS OF STEROIDS IN ACTION IN THE CONTEXT OF PREGNANCY

Attracting spermatozoids by progesterone

In order to get pregnant an oocyte needs to be fused with one spermatozoid. In humans, sperm is normally “deposited” in the anterior vagina (near the fornix vaginae) and from there they have to cross the cavum

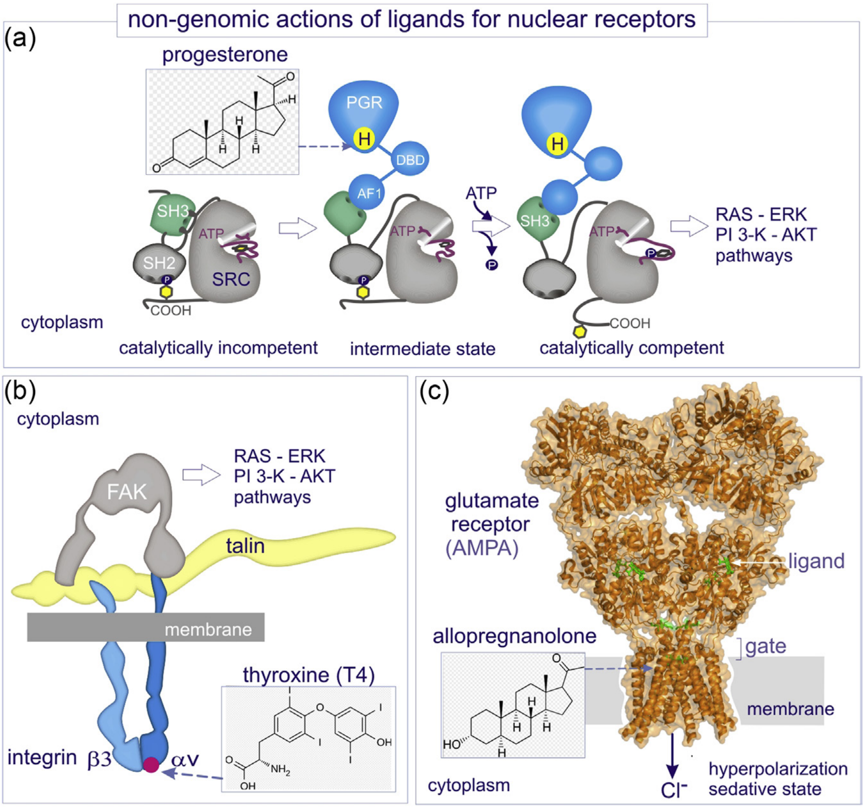


FIGURE 8-14 **Three examples of non-genomic actions of steroid and thyroid hormones. The details of the pathway are not discussed in this chapter.** (a) The progesterone receptor contains a proline-rich region in the AF-1 segment which can interact with the SH3 domain of tyrosine kinases SRC. This removes an important inhibitory constraint and facilitates the full activation process. (b) Thyroxine (T4) binds to the ligand-binding pocket of the adhesion molecule integrin β3αv and augments the activity of the focal adhesion protein kinase FAK. (c) Allopregnanolone inserts into the gate of the AMPA-type (ionotropic) glutamate receptors and enhances conductance for Cl^- (by prolonging the open state).

uteri in the direction of the utero-tubal junction (a distance of ~6 cm). Upon arrival in the isthmus of the fallopian tube (only a few thousands reach this point out of ~60 million or so deposited) they attach to the epithelium (via a lectin–fucose bond) which leads to the formation of a sperm reservoir. In order to continue the spermatozoids have to undergo a process of capacitation which is defined as those physiological events that render them able to fertilize. It comprises hyperactivation and the acrosome reaction. Hyperactivation is characterized by a high amplitude and asymmetrical flagellar beat which is required for the penetration of the cumulus cell layer that surrounds the oocyte. The acrosome reaction is the process in which a huge secretion vesicle, named acrosome, fuses with the plasma membrane at numerous sites thus liberating vesicle content and bringing novel transmembrane proteins to the cell surface. The acrosome reaction is vital for fertilization but how it contributes remains an enigma (the hyaluronidase and protease activity apparently are dispensable) (Figure 8-15) (Coy et al., 2012; Buffone et al., 2014).

The capacitation signal is an increase in intracellular Ca^{2+} which enters the spermatozoid through a voltage-sensitive anion channel named CATSPER (CATSPER1-4). It is located in the principal piece of the flagellum and indeed male mice lacking this channel are infertile (Ren et al., 2001; Kirichok et al., 2006). The channel resembles a voltage-gated ion channel with six transmembrane passages organized in four repeats (return to Figure 6-12, example of TRPC) and is permeable to both Ca^{2+} and Na^+ but with preference for the first. The question remains: "what activates Ca^{2+} entry?"

CATSPER is sensitive to cAMP but this is not essential for activation in the fallopian tube. It was shown that CATSPER is activated by the combined action of progesterone, released by cumulus cells, and an elevation of the intracellular pH through entry of HCO_3^-. Progesterone either directly binds to the channel, or to a regulatory subunit, and increases anion conductivity (Ca^{2+} currents) (Strunker et al., 2011; Lishko et al., 2011). The entry of Ca^{2+} may bring about a cascade of Ca^{2+}-induced Ca^{2+} release from intracellular stores and the ensuing Ca^{2+} waves will reach the acrosome causing fusion with the plasma membrane at multiples sites (return to Chapter 6 "Intracellular Calcium", Figure 6-20). How Ca^{2+} influences flagellar movement is not clear to us. Remember that flagellar movement involves the tubulin cytoskeleton (organized in an axoneme) associated with dynein motor proteins which, unlike smooth muscle myosine, do not necessarily require Ca^{2+}. We note that, in sea urchin sperm, cilia of scallop and *chlamydomonas* cells, calmodulin co-purifies with radial-spoke proteins and with a small regulatory subunit of dynein (LC8) (Stommel et al., 1982; Yang et al., 2001). In sea urchin sperm, Ca^{2+} was shown to control axonemal curvature in the flagellar waveform (giving rise to a high-amplitude asymmetrical beat) and that this process requires CAMK2 (Rokaw et al., 1974; Smith, 2002).

Sperm capacitation is essential for both in vivo and in vitro fertilization. The phenomenon was recognized by Patrick Steptoe, NHS Royal Oldham Hospital, UK, and effectively exploited in collaboration with Robert Edwards, University of Cambridge, UK, in his pursuit of in vitro fertilization (IVF). The first IVF baby was born in the Royal Oldham Hospital in 1978. Edwards was awarded the Nobel Prize in Physiology or Medicine in 2010 "for the development of in vitro fertilization" (Steptoe was already deceased).

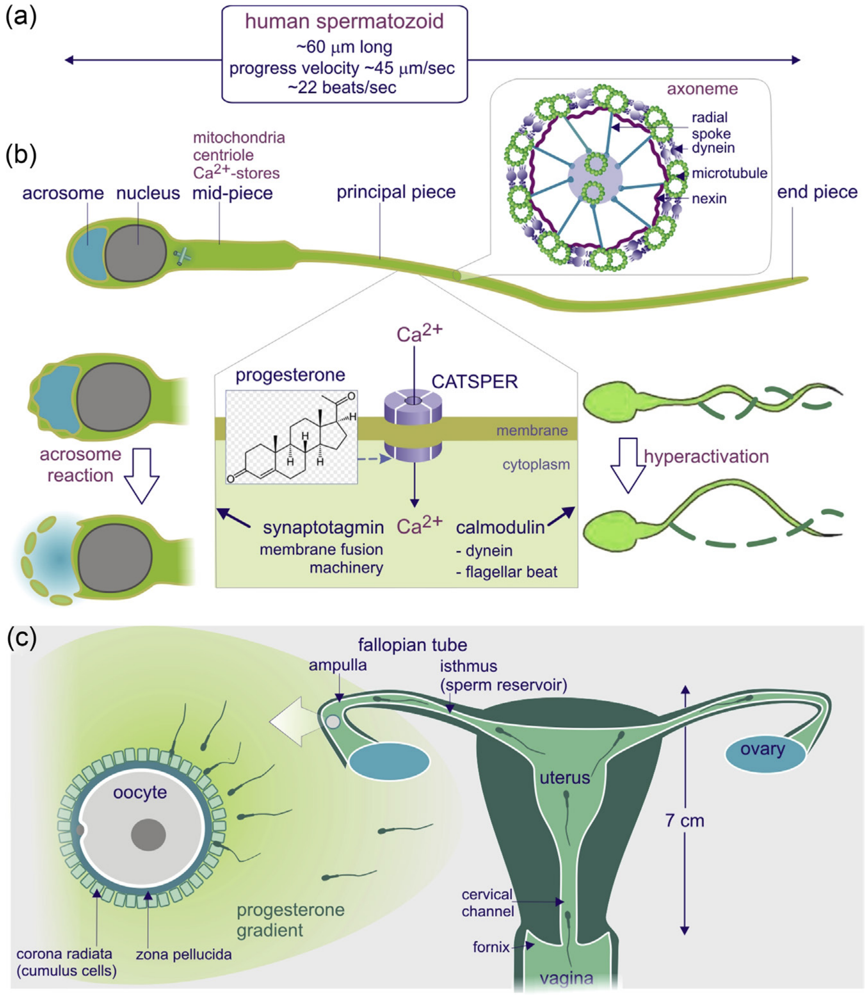


FIGURE 8-15 Sperm capacitance and the non-genomic action of progesterone. (a) Anatomy of a human spermatozoid. The acrosome contains enzymes (proteases and hyalorunidase), the midpiece houses the centriole (nucleation site of microtubules), numerous mitochondria and Ca^{2+}-storing organelles. The tail contains a core axoneme, a typical microtubule structure combined with dynein motor proteins. (b) Progesterone, produced by cumulus cells, enhances Ca^{2+} conductivity either by direct binding to the CATSPER channel or to an as yet undefined regulatory subunit. Ca^{2+} entry brings about sperm capacitance, a process comprising hyperactivation and the acrosome reaction. The increase in intracellular Ca^{2+} causes a change in the flagellar beat, going from a symmetric low-amplitude to an asymmetric high-amplitude movement (hyperactivation). Calmodulin plays a role but the mechanism is not fully understood. Ca^{2+} also interacts with and stimulates the membrane fusion machinery, probably through synaptotagmin and the acrosome fuses with the plasma membrane at multiple sides (acrosome reaction). (c) Sperm capacitation occurs at the level of isthmus of the fallopian tube, where sperm accumulates in preparation for the final assault on the oocyte which is lodged in the ampulla. Progesterone, released by cumulus cells of the corona radiate, plays an important role. Near the oocyte, the spermatozoids have to breach a barrier made up of cells and the zona pellucida. Capacitation is necessary for fertilization but what exactly is important remains elusive (proteolytic and hyaluronidase activity apparently are dispensable in mice).

Regulation of mammary gland stem-cell proliferation through paracrine signaling

Estrogen and progesterone are produced transiently during the menstrual cycle and continuously during pregnancy where it is first produced by the corpus luteum, under the influence of HCG, and then by the placenta. The high circulating levels of these hormones feed back suppression of the pituitary and prevent ovulation. Progesterone production in the luteal phase is important as it allows for successful embryo attachment (nidation) and endometrium development, a process referred to as decidualization. It is characterized by increased glandular differentiation, recruitment of eosinophils and natural killer cells in perivascular space, increased glandular secretion, glycogen accumulation, and stromal vascularity and edema. During pregnancy, estrogen and progesterone play a role in the full development of milk-secreting mammary glands, the maintenance of the endometrium, and progesterone helps to suppress accidental uterine contractions.

The mammary gland, made up of alveoli and ducts, is normally poorly developed but nevertheless a dynamic organ (after puberty). With each estrus cycle (menstrual cycle for humans), the epithelium enters a phase of proliferation and differentiation, leading to the emergence of small alveolar buds, followed by cell loss (involution). Important long lasting expansion occurs during pregnancy. It is generally considered that estrogen drives ductal elongation which causes the infiltration of the gland into the fat pads (adipose tissue of the breast), whereas progesterone contributes to ductal side branching and alveologenesis (Figure 8-16). Prolactin too plays a role in mammary gland development and lactation, but at a later stage in pregnancy (Joshi et al., 2012).

Mammary gland stem cells (often indicated as MaSC) have been discerned in the basal and luminal layers (Figure 8-17), in mouse and human, and they are particularly active in the later phase of the estrus cycle (diestrus, when progesterone is high) and during pregnancy. Stem cell numbers increase transiently during these two phases. In mice they are distributed throughout the gland but may be restricted to terminal ducts in humans. Only the stem cells from the basal layer (characterized by high integrin-β1α6 and moderate CD24 expression), when isolated and inoculated into cleared mammary fat pads of mice, are capable of the formation of both basal (myoepithelial) and luminal (secretory epithelial cells) lineages and generate functional lobuloalveolar units during pregnancy (Kordon and Smith, 1998; Stingl et al., 1998; Shackleton et al., 2006). Progesterone receptors are uniformly expressed in all epithelial cells in juvenile mammary glands but expression switches to a heterogeneous pattern during puberty and in the adult. The remaining (~40%) receptor-positive cells are non-proliferative. The spatial segregation also applies to estrogen receptors.

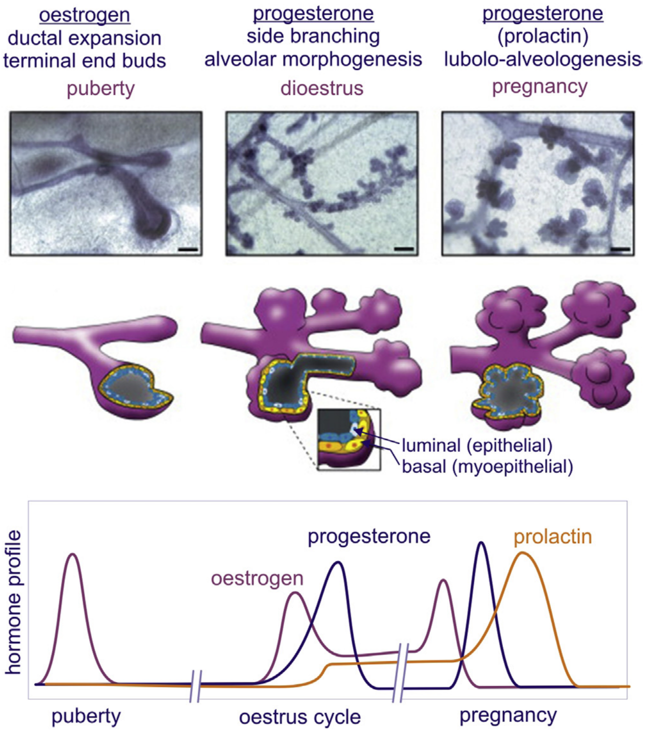


FIGURE 8-16 Steroid hormone-controlled mammary-gland development in the mouse during puberty, diestrus (luteal phase), and pregnancy. *Image is adapted with permission from Joshi et al. (2012).*

Surprisingly, the mammary stem-cell population resides in the receptor negative population (for both hormones). If estrogen/progesterone-receptor bearing cells are not proliferative, how could these steroids play a role in gland development? The answer lies in a paracrine mode of cell–cell communication.

There are some controversies as to whether the proliferating cells in the luminal layer are true stem cells. They may represent early stage progenitor cells. It is clear that only basal stem cells give rise to functional glands in explant experiments, but a recent study has shown that in situ, both basal and luminal cells also originate from basal stem cells (lineage analysis in mice with fluorescent cell markers) (Rios et al., 2014). This brings into question if luminal cells harbor true stem cells.

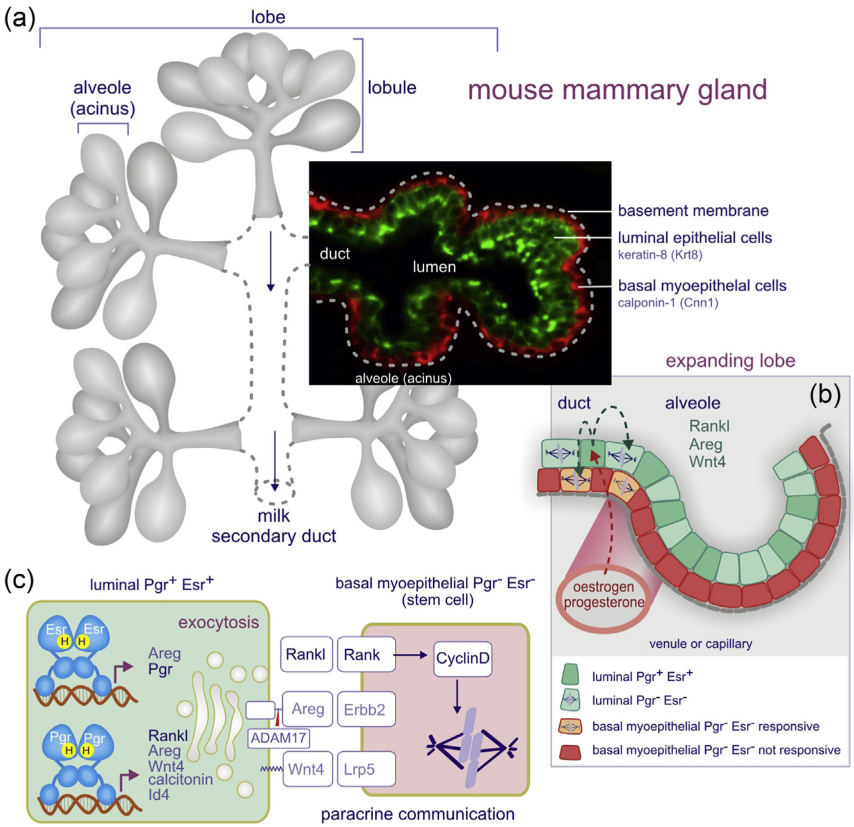


FIGURE 8-17 **Paracrine signaling between estrogen and progesterone receptor-bearing epithelial cells and mammary-gland stem cells.** (a) Structure of a mammary gland lobe with ducts, lobules, and alveoli. The epithelial layer is composed of basal (myoepithelial) and luminal (secretory epithelial) cells, stained for smooth muscle marker calponin-1 and the keratin-8, respectively. The image of the immunostained alveaole is kindly provided by Marina Gloukhova, Insitut Curie, Paris, France. (b) Estrogen and progesterone enter the gland from the blood and bind to luminal progesterone- and estrogen-receptor positive cells (Pgr$^+$, Esr$^+$). They induce gene transcription and provide local paracrine cues such as Rankl, Areg, and Wnt4. Only a fraction of the basal and luminal cells are responsive and start to proliferate to give rise to a new stem cell and a progenitor cell. The progenitor cell rapidly divides before the progeny differentiates into one of the two cell types. Progenitor cell expansion is particularly important during pregnancy. (c) The occupied estrogen receptor induces expression of amphiregulin (Areg) and the progesterone receptor. The first acts as an EGF-like growth factor on adjacent cells, the other primes the cell for the progesterone signal. The occupied progesterone receptor induces expression of Rankl, Areg, Wnt4, calcitonin, and Id4. Of these, Rankl was found to be highly expressed during pregnancy in mice. It binds to adjacent basal cells (or luminal cells) and induces their proliferation (increase in the production of cyclin D among many other events). Adjacent stem cells also express receptors for Areg and Wnt4 and they too play a role in the orchestration of the developing mammary gland. Areg is a transmembrane protein which is cleaved by the protease ADAM17. Wnt4 has a lipid tail and only diffuses slowly into the tissue.

Addition of estrogen was shown to induce expression of amphiregulin and the progesterone receptor, whereas progesterone induces expression of Id4, calcitonin, amphiregulin (AREG), Wnt4, and RANKL (among other genes) (Figure 8-17). These are genomic responses induced by nuclear receptors. Expression of these genes occurs in ER^+PR^+ luminal epithelial cells of which some are located next to mammary gland stem cells. Besides Id4 (which is a DNA-binding protein) and calcitonin (which is a hormone that protects against bone loss during pregnancy) these genes code for cytokines with growth-promoting capacities for mammary epithelial cells (Obr and Edwards, 2012). Mammary gland stem cells express the receptor for all three: ERRB2 (amphiregulin), Lrp5 (Wnt4), and RANK (RANKL). Of these genes, expression of RANKL is particularly elevated during pregnancy in mice and it was shown that its inhibition (through an antibody) significantly reduced clonogenic activity of stem cell-enriched cell populations (Asselin-Labat et al., 2010). Activation of RANK, the receptor, by its ligand signals the expression of cyclin D1 (CND1) which is a regulatory component of the cell cycle kinases-4 and -6 that drive cell-cycle progression, thereby enhancing the division rate. The stem cells give rise to progenitor cells which divide rapidly before they differentiate into basal or luminal cells. The progenitor cells play an important role in the expansion of the gland during pregnancy (more so than during the menstrual cycle) (Joshi et al., 2012).

RANKL AND METASTASIS

Regulatory T-cells that infiltrate mammary gland carcinomas produce RANKL and this enhances lung metastasis by the tumor cells (Tan et al., 2011). Besides the lungs, bones also are frequently targeted by invasive mammary gland cells. Here the tumor cells induce the expression of RANKL in osteoblasts and this has two nasty consequences; it leads to the differentiation of osteoclasts, giving rise to bone loss (osteolysis) and it further stimulates tumor cell growth (Yoneda et al., 2013).

Besides surgical intervention and chemotherapy, the treatment of breast cancer may also include efforts to reduce the action of estrogen. In premenopausal women the mean sources of estrogen are the ovaries whereas in postmenopausal, when ovaries have seized their production, dominant sources are the central nervous system, the dermis, and adipose tissue (Nelson, 2001). Currently three approaches are employed. The first concerns inhibition of the production of estrogen by aromatase inhibitors. These block the enzyme (CYP19A1) which normally converts androstenedione into estrone and testosterone into beta-estradiol. Examples of inhibitors are exemestane (Aromasin), anastrozole (Arimidex), and letrozole (Femara). The second concerns selective estrogen-receptor modulators (SERM), including antagonists such as tamoxifen (Novadex), raloxifen (Evista), or toremifene (Farestone). The third approach also involves antagonists but those that have a higher propensity to cause degradation of the estrogen receptor hence they are named estrogen receptor down-regulators. One example: fulvestrant (Faslodex).

Web resource

Breast cancer organization http://www.breastcancer.org.

Consolidating memories with cortisol

Finding out to be pregnant certainly ranks among the major emotional events in life. The news, by itself a brief statement of a gynecologist or a predictor test, will be consolidated in a long-lasting memory. The memory plays an essential role in dealing with the new condition (taking decisions for the future). News laden with emotion activates the hypothalamic–pituitary–adrenal axis, starting with the release of corticotropin-releasing hormone (CRH) in the hypothalamus, followed by release of adrenocorticotropic hormone (ACTH) in the anterior pituitary, and finally the release into the blood of cortisol from the cortex of the adrenal gland (and corticosterone in most rodents). The signal immediately returns to the brain where cortisol mediate and modulate memory consolidation. Regions of the brains concerned are the hippocampus, amygdala, and prefrontal cortex, each of which express cortisol receptors (both gluco- and mineralcorticoids (NR3C1 and -2)). It is important to notice that cortisol binds with higher affinity to the mineralcorticoid (aldosterone) receptor (NR3C2) than to its “cognate” glucocorticoid receptor (NR3C1). Although both receptors attach to identical response elements and theoretically bring about similar responses in a given cell (return to Figure 8-4), in reality they cause different effects due to post-translational modifications. Low doses of cortisol thus mobilize the mineral corticoid receptor in the brain whereas higher doses involve both receptor types. Memory consolidation related to improved cognitive performance is thought to act through both the mineralcorticoid and glucocortocid receptors (Joels et al., 2008).

The process of long-term memory formation requires a change in networking of neurons which means the formation of new synapses or the reinforcement of existing ones. With reinforcement we refer to the phenomenon that a single release of glutamate causes an enhanced excitatory post-synaptic potential. The calcium–calmodulin-sensitive protein kinase-IIα (CAMK2A) is a key in this phenomenon and in the context of glucocorticoid-enhanced learning it receives two signals both through non-genomic actions of cortisol (Figure 8-18). The first originates from an enhanced glucocorticoid-induced Ca^{2+} influx through an as yet undefined mechanism (enhanced conductance of an L-type voltage sensitive Ca^{2+} channel or of the glutamate NMDA receptor) (Chameau et al., 2007; Takahashi et al., 2002). The second occurs through a glucocorticoid-mediated transactivation of the BDNF receptor (TrkB, formal nomenclatureNTRK2), a tyrosine-kinase receptor type, which through activation of PLCγ (PLCG) causes Ca^{2+} release from intracellular stores (return to Chapter 6 "Intracellular Calcium"). Both pathways converge on CAMKII-α. Its activation results in the recruitment of the AMPA-receptor in the synaptic cleft and in phosphorylation of CREB in the nucleus. CREB joins the glucocorticoid receptor NR3C2 in the nucleus and together, by binding to their respective response elements, they bring about a change in gene expression that will consolidate synapse "strengthening" (Chen et al., 2012; Finsterwald and Alberini, 2014).

PRE-RECEPTOR STEROID METABOLISM GOVERNS RECEPTOR SPECIFICITY

Glucocorticoid and mineralcorticoid receptors share a high affinity for both classes of steroids but differ in their binding kinetics. The mineral corticoid receptor (NR3C1) constitutes a high-affinity, low-capacity corticosteroid-binding site (K_D 0.5–3 nM) for both aldosterone and cortisol. Glucocorticoid receptors offer a low-affinity, high-capacity binding site (K_D 20–65 nM) for both steroids. The bioavailability thus plays an important role in response selectivity. Kidneys express 11β-hydroxysteroid hydrogenase type-2 which converts cortisol (or corticosterone in rodents) into inactive 11-keto derivatives (Chapman et al., 2013). The depletion is not complete and an excess of cortisol, as occurs in anti-inflammatory therapy or pituitary cancer, causes hypertension because it mimics the action of aldosterone (Na^+ retention).

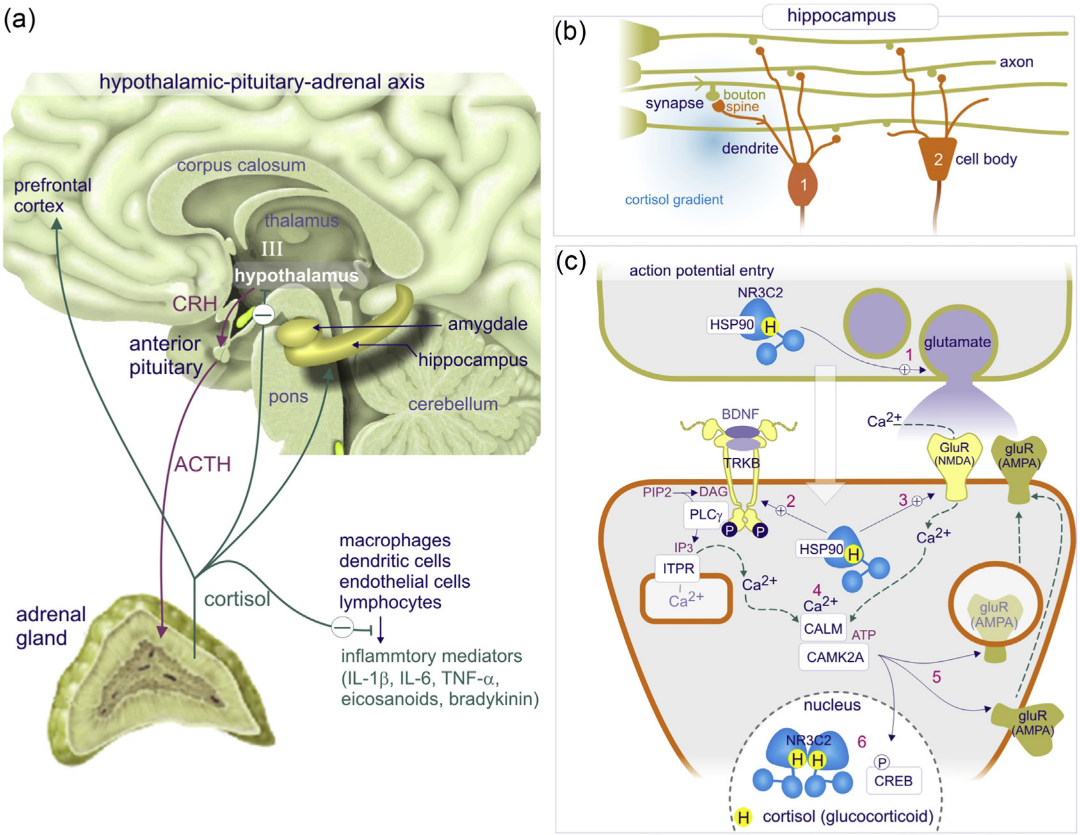

(a)
hypothalamic-pituitary-adrenal axis
corpus calosum
prefrontal cortex
thalamus
III
hypothalamus
CRH
anterior pituitary
amygdale
hippocampus
pons
cerebellum
ACTH
macrophages
dendritic cells
endothelial cells
lymphocytes
cortisol
adrenal gland
inflammtory mediators
(IL-1β, IL-6, TNF-α,
eicosanoids, bradykinin)
(b)
hippocampus
axon
synapse
bouton
spine
dendrite
1
2
cell body
cortisol gradient
(c)
action potential entry
NR3C2
HSP90
H
1
glutamate
BDNF
Ca2+
TRKB
GluR (NMDA)
gluR (AMPA)
PIP2
DAG
PLCγ
P
2
3
IP3
ITPR
HSP90
Ca2+
4
CALM
ATP
CAMK2A
5
gluR (AMPA)
nucleus
NR3C2
6
P
CREB
cortisol (glucocorticoid)

FIGURE 8-18 **Glucocorticoid-mediated memory consolidation.** (a) Inflammation or news laden with emotion activates the hypothalamic–pituitary–adrenal axis which starts with the release of corticotropin-releasing hormone (CRH) in the hypothalamus, followed by release of adrenocorticotropic hormone (ACTH) in the anterior pituitary and finally the release into the blood of cortisol (glucocorticoid) from the cortex of the adrenal gland. Cortisol feeds back on immune cells, holding inflammation in check, and on different anatomical structures of the brain (amygdale, hippocampus, and prefrontal cortex). (b) The hippocampus plays an important role in memory consolidation. The process involves the strengthening of synaptic contacts between neurons. As shown here, one horizontal axon has developed and enlarged synapse (enlarged bouton) with one other neuron (enlarged spine). As a consequence a signal will pass by neuron 1 and not by neuron 2. (c) Synapse strengthening occurs through an enhanced signal transmission caused by an enhanced release of excitatory neurotransmitters (glutamate in the example) and an increase in the number of ionotropic receptors (the AMPA-type glutamate receptor in the example). Non-genomic actions of the cortisol, through binding to the mineralcorticosteroid receptor NR3C2, play an important role in the reenforcement of synaptic contact. (1) In the pre-synaptic neuron it causes enhanced release of glutamate. In the post-synaptic neuron it causes (2) activation of TRKB, a tyrosine protein kinase receptor that binds the brain-derived neurotrophic factor BDNF. This leads to activation of phospholipase-Cγ, giving rise to the production of IP_3 and the release of Ca^{2+} from intracellular stores. (3) Cortisol also enhances conductivity of the NMDA receptor and both Ca^{2+} entry pathways contribute a role in (4) the activation of the Ca^{2+}–calmodulin-dependent protein kinase-2α (CAMK2A). This in turn facilitates the recruitment (5) of AMPA receptors into the synaptic cleft and (6) to phosphorylation of the transcription factor CREB in the nucleus. Both the NR3C2 and CREB contribute to gene expression patterns that further consolidate the synaptic strength (and the memory it bears).

GLUCOCORTICOIDS AND COGNITIVE IMPAIRMENT

Chronic stress and single severely traumatic experiences can have negative impact on cognitive functions and lead to the development of several psychopathologies. Stress and cognitive performance (of complex tasks) follow an inverted U-shaped curve. With an increase in stress intensity, cognitive performance enhances until it reaches a maximum and then declines. It is believed that glucocorticoid concentrations rise with stress intensity and that too much glucocorticoids block cognitive performance.

LONG-TERM POTENTIATION

The glutamate AMPA receptors (heterotetramer of GRIA1, -2) play a role in response adaptation in synapses in the process known as long-term potentiation (LTP). There are two essential components; presynaptic glutamate release and postsynaptic depolarization. The process can be observed in a paired electrophysiological recording, when a presynaptic cell is stimulated to release glutamate on a postsynaptic cell that is depolarized. In a typical learning protocol a "tetanus" stimulation is applied at 100 Hz for 1 s in a presynaptic pathway. As a consequence a sustained increase in the excitatory postsynaptic potential is observed with subsequent electrical stimulation. In other words the chance that a single stimulus elicits an action potential in the post-synaptic neuron is increased. LTP is thought to be the physiological correlate for learning and memory in the cell. In fact, it was recently shown that, following a single paired-avoidance protocol in mice, LTP could be recorded in some hippocampal synapses in vivo (Whitlock et al., 2006). Increased AMPA-receptor expression and their recruitment in the synaptic area are two postsynaptic molecular mechanisms underlying the above described phenomenon. In addition, local enhanced translation of β-actin, through unmasking of the mRNA, plays an essential role in altering the synapse morphology (increased surface and outgrowth of bouton and spine). The process requires NMDA receptors (Ca^{2+} influx) and members of the MAPKK family of protein kinases (Buxbaum et al., 2014).

Multimedia resource

http://en.wikipedia.org/wiki/Long-term_potentiation.

ENDOCRINE DISRUPTION IN A PLASTIC WORLD: THE EXAMPLE OF BISPHENOL A

Bisphenol A is a key monomer in production of epoxy resins and in the most common form of polycarbonate plastic (plastic bottles, lining of metal food and beverage cans, lining of hulls of modern composite vessels, or laboratory centrifuge tubes). Several studies have shown that bisphenol A is released from consumer goods, leading to its detection in food, drinking water, wastewater, soil, dust, and air (Dekant and Volkel, 2008). Bisphenol binds the estrogen receptor and causes it to adopt the canonical active conformation; with helix H12 capping the ligand-binding pocket and attachment of the nuclear receptor co-activator NCOA1 (Delfosse et al., 2012). Bisphenol A therefore qualifies as a xenoestrogen. It affects mammary-gland development in mice and it may play a role in early onset of human obesity or other metabolic syndromes (Rezg et al., 2014). Apart from the estrogen receptors, bisphenol A is also recognized by the pregnane receptor (NR1I2), peroxisome proliferator-activated receptor-γ (PPARG), and the estrogen receptor-related receptor-γ (ESRRG). The latter is highly expressed in the placenta and makes the tissue particularly vulnerable (Vandenberg et al., 2007). It is important to notice that the xenoestrogenic action is not necessarily exerted in all cells and effects should be tested on numerous different tissues. There is no ban on bisphenol A but governments are cautious and urge industry to search for alternatives.

References

Addison, T., 1855. On the Constitutional Local Effects of Disease of the Suprarenal Capsules. P. Highley, London.

Aronica, S.M., Kraus, W.M., Katzenellenbogen, B.S., 1994. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc. Natl. Acad. Sci. U.S.A. 91, 8517–8521.

Ashburner, M., Bonner, J.J., 1979. The induction of gene activity in Drosophila by heat shock. Cell 17, 241–254.

Asselin-Labat, M.-L., Vaillant, F., Sheridan, J.M., Pal, B., Wu, D., Simpson, E.R., Yasuda, H., Smyth, G.K., Martin, T.J., Lindeman, G.J., Visvader, J.E., 2010. Control of mammary stem cell function by steroid hormone signalling. Nature 210 (465), 798–802.

Baker, M.E., 2003. Evolution of adrenal and sex steroi action in vertebrates: a ligand-based mechanism for complexity. Bioessays 25, 396–400.

Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., Beato, M., 2003. Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-SRC-ERK pathway in mammalian cells. Mol. Biol. Cell 23, 1994–2008.

Beatson, G.T., 1896. On the treatment of inoperable cases of carcinoma of the mammary: suggestions for a new method of treatment with illustrative cases. Lancet 2, 104–107.

Bergh, J.J., Lansing, L., Mohamed, S.N., Davis, F.B., Mousa, S., Davis, P.J., 2005. Integrin αvβ3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology 146, 2864–2871.

Boonyaratanakornkit, V., Porter-Scott, M., Ribon, V., Sherman, L., Anderson, S.M., Maller, J.L., Miller, W.T., Edwards, D.P., 2001. Progesterone receptor contains a proline-rich motif that directly interacts with SH3-domains ad activates c-Src family tyrosine kinases. Mol. Cell 8, 269–280.

Buffone, M.G., Hirohashi, N., Gerton, G.L., 2014. Unresolved questions concerning mammalian sperm acrosomal exocytosis. Biol. Reprod. 90, 112.

Bunone, G., Brian, P.-A., Miksicek, J., Picard, D., 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. 15, 2174–2183.

Butenandt, A., Westphal, U., Hohlweg, W., 1934. Über das Hormon des Corpus luteum. Hoppe-Seylerś Zschr. Physiol. Chem. 227, 84–98.

Buxbaum, A.R., Wu, B., Singer, R.H., 2014. Single β-actin mRNA detection in neurons reveals a mechanism for regulating its translatability. Science 343, 419–422.

Chameau, P., Qin, Y., Spijker, S., Smit, G., Joels, M., 2007. Glucocorticoids specifically enhance L-type calcium current amplitude and affect calcium subunit expression in the mouse hippocampus. J. Neurophysiol. 97, 5–14.

Chapman, K., Holmes, M., Seckl, J., 2013. 11β–hydroxysteroid dehydrogenases: intracellular gate–keepers of tissue glucocorticoid action. Physiol. Rev. 93, 1139–1206.

Chen, D.Y., Bambah-Mukku, D., Pollonini, G., Alberini, C.M., 2012. Glucocorticoid receptors recruit the CAMKAA-a-BDNF-CREB pathways to mediate memory consolidation. Nat. Neurosci. 15, 1707–1714.

Clever, U., Karlson, P., 1960. Induktion von puff-veränderungen in den speicheldrüsenchromosomen von Chironmus tentans durch ecdyson. Exp. Cell Res. 20, 623–626.

Cosma, M.P., 2002. Ordered recruitment: gene-specific mechanism of transcription activation. Mol. Cell 10, 227–236.

Cottrell, G.A., Lambert, J.J., Peters, J.A., 1987. Modulation of GABAA receptor activity by alphaxalone. Br. J. Pharmacol. 90, 491–500.

Coy, P., Garcia-Vasquez, F.A., Visconti, P.E., Avilés, M., 2012. Roles of oviduct in mammalian fertilization. Reproduction 144, 649–660.

David, K.G., Dingemanse, E., Freud, J., Laqueur, E., 1935. Über krystallinisches mannliches hormon aus hoden (testosteron) wirksamer als aus harn oder aus cholesterin bereitetes androsteron" [On crystalline male hormone from testicles (testosterone) effective as from urine or from cholesterol]. Hoppe Seylers Z. Physiol. Chem. 233, 281.

de Duve, C., 1969. The peroxisome: a new cytoplasmic organelle. Proc. R. Soc. Lond., B, Biol. Sci. 173, 71–83.

Dekant, W., Volkel, W., 2008. Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicol. Appl. Pharamcol. 228, 114–134.

Delfosse, V., Grimaldi, M., Pons, J.-L., Boulahtouf, A., le Maire, A., Cavailles, V., Labesse, G., Bourguet, W., Balaguer, P., 2012. Structural and mechanistic insight into bisphenols action provide guidelines for risk assessment and discocery of bisphenol A substitutes. Proc. Natl. Acad. Sci. U.S.A. 109, 14930–14935.

Doisy, E.A., Veler, C.D., Thayer, S., 1930. The preparation of the crystalline ovarian hormone from the urine of pregnant women. J. Biol. Chem. 86, 499–509.

Doisy, E.A., 1976. An autobiographie. Ann. Rev. Biochem. 45, 1–12.

Dreyer, C., Krey, G., Keller, H., Givel, G., Helftenbein, G., Wahli, W., 1992. Control of peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68, 879–887.

Escriva, H., Delaunay, F., Laudet, V., 2003. Ligand binding and nuclear receptor evolution. Bioessays 22, 717–727.

Finsterwald, C., Alberini, C.M., 2014. Stress and glucocorticoid receptor-dependent mechanisms in long-term memory: from adaptive response to psychpathologies. Neurobiol. Learn Mem. 112, 17–29.

Frobenius, W., 1998. Ludwig Fraenkel, corpus luteum and discovery of progesterone. Zentralbl. Gynakol. 120, 317–323.

Germain, P., Iyer, J., Zecher, C., Gronemeyer, H., 2002. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. Nature 415, 187–192.

Ghisletti, S., Huang, W., Ogawa, S., Pascual, G., Lin, M.E., Willson, T.M., Rosenfeld, M.G., Glass, C.K., 2007. Parallel SUMOylation-depedent pathways mediate gene- and signal-specific transrepression by LXRs and PPARγ. Mol. Cell 25, 57–70.

Glascock, R.F., Hoekstra, W.G., 1957. Selective accumulation of tritium-labeled heoxestrol by the reproductive organs of immature female goats and sheep. Biochem. J. 72, 673–682.

Glass, C.K., Saijo, K., 2010. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. Nat. Rev. Immunol. 10, 365–376.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, O., Chambon, P., 1986. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320, 134–139.

Greenstein, S., Ghias, K., Krett, N.L., Rosen, S.T., 2002. Mechanism of glucocorticoid-mediated apoptosis in haematological malignancies. Clin. Cancer Res. 8, 1681–1694.

Gronemeyer, H., Gustafsson, J.-A., Laudet, V., 2004. Principles for modulation of the nuclear receptor superfamily. Nat. Rev. Drug Discov. 3, 950–964.

Grundy, H.M., Simpson, S.A., Tait, J.F., 1952. Isolation and a highly active mineralcorticoid from beef adrenal extract. Nature 169, 795–796.

Grygiel-Gorniak, B., 2014. Peroxisome proliferator-activated receptors and their ligands: nutrinional and clinical implications—a review. Nutr. J. 13, 17.

Hartmann, M., Wettstein, A., 1934. Ein krystallisiertes hormon aus dem corpus luteum. Helv. Chim. Acta 17, 878–882.

Hendrick, J.P., Hartl, F.-U., 1993. Molecular chaperone functions of heat-shock proteins. Ann. Rev. Biochem. 62, 349–384.

Hernanson, O., Glass, C.K., Rosenfeld, M.G., 2002. Nuclear receptor co-regulators: multiple modes of modification. Trends Endocrin. Metab. 13, 55–60.

Hollenberg, S.M., Weinberger, C., Ong, E.S., Cerelli, G., Oro, A., Lebo, R., Thompson, E.B., Rosenfeld, M.G., Evans, M.R., 1985. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318, 635–641.

Huang, P., Chandra, V., Rastinejad, F., 2010. Structural overview of the nuclear receptor superfamily: insight into physiology and therapeutics. Annu. Rev. Physiol. 72, 247–272.

Hudson, W.H., Youn, C., Orlund, E.A., 2013. The structural basis of direct glucocorticoid-mediated transrepression. Nat. Struct. Mol. Biol. 20, 53–58.

Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347, 645–650.

Jiang, C., Ting, A., Seed, B., 1998. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Joels, M., Karst, H., de Rijk, R., de Kloet, E.R., 2008. The coming out of the brain mineralcorticoid receptor. Trends Neurosci. 31, 1–7.

John, S., Sabo, P.J., Thruman, R.E., Sung, M.H., Biddie, S.C., Johnson, R.A., Hager, G.L., Stamatoyannopoulus, J.A., 2011. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nat. Genet. 43, 264–268.

Joshi, P.A., Di Grappa, M.A., Khokha, R., 2012. Active allies: hormones, stem cells and the niche in adult mammopoiesis. Trends Endocrinol. Metabol. 6, 299–309.

Kassel, O., Herrlich, P., 2007. Crosstalk between glucocorticoid receptor and other transcription factors: molecular aspects. Mol. Cell Endocrinol. 275, 13–29.

Kendall, E.C., December 11, 1950. The Development of Cortisone as a Therapeutic Agent. Nobel Lecture.

Kirichok, Y., Navarro, B., Clapham, D.E., 2006. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca^{2+} channel. Nature 439, 737–740.

Kleiman, A., Hubner, S., Parkitna, J.M.R., Neumann, A., Hoferr, S., Weigand, M.A., Bauer, M., Schmid, W., Schutz, G., Libert, C., Reichard, H.M., Tuckermann, J.P., 2012. Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. FASEB J. 26, 722–729.

Klein, K., Henk, W., 1963. Klinisch-experimentelle untersuchungen uber den einflus von aldosterron auf hämodynamik and gerinnung. Z. Kreisl. Forsch. 52, 40–53.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Jones, M.A., McKee, D.D., Oliver, B.M., Willson, T.M., Zetterstrom, R.H., Perlmann, T., Lehmann, J., 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 92, 73–82.

Kordon, E.C., Smith, G.H., 1998. An entire functional mammary gland may comprise the progeny from a single cell. Development 125, 1921–1930.

Kostrouch, Z., Kostrouchova, M., Love, W., Jannini, E., Piatigorsky, J., Rall, J.E., 1998. Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. Proc. Natl. Acad. Sci. U.S.A. 95, 13442–13447.

Laudet, V., 1997. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. J. Mol. Endocrinol. 19, 207–226.

Le, W.-D., Xu, P., Jankovic, J., Appel, S.H., Smith, R.G., Vassilatis, D.K., 2002. Mutations in NR4A2 associated with familial Parkinson disease. Nat. Genet. 33, 85–89.

Le Mellay, V., Liberherr, M., 2000. Membrane signaling and progesterone in female and male osteoblasts. II. Direct involvement of Gαq11 coupled to PLCβ1 and PLCβ3. J. Cell Biochem. 79, 173–181.

Lishko, P.V., Botchkina, I.L., Kirichok, Y., 2011. Progesterone activates the principal Ca2+ channel of human sperm. Nature 471, 387–391.

Lorenz, O.R., Freiburger, L., Rutz, D.A., Krause, L., Zierer, B.K., Alvira, S., Cuellar, J., Valpuesta, J.M., Madl, T., Sattler, M., Buchner, J., 2014. Modulation of the Hsp90 chaperone cycle by a stringent client protein. Mol. Cell 53, 941–953.

Lösel, R., Wehling, M., 2003. Nongenomic actions of steroid hormones. Nat. Rev. Mol. Cell Biol. 4, 46–56.

le Maire, A., Teyssier, C., Erb, C., Grimaldi, M., Alvarez, S., de Lera, A.R., Balguer, P., Gronemeyer, H., Royer, C.A., Germain, P., Bourguet, W., 2010. A unique secondary-structure switch controls constitutive gene repression by retinoic acid receptor. Nat. Struct. Mol. Biol. 17, 801–807.

Meijsing, S.H., Pufall, M.A., So, A.Y., Bates, D.L., Chen, L., Yamamoto, K.R., 2009. DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324, 407–410.

Metivier, R., Penot, G., Carmouche, R.P., Hubner, P.R., Reid, G., Denger, S., Lan, D., Brand, H., Kos, M., Benes, V., Gannon, F., 2004. Transcriptional complexes engaged by apo-estrogen receptor-a isoforms have divergent outcomes. EMBO J. 23, 3653–3666.

Moore, L.B., Parks, D.J., Jones, S.A., Bledsoe, R.K., Consler, T.G., Stimmer, J.B., Goodwin, B., Liddle, C., Blanchard, S.G., Willson, T.M., Collins, J.L., Kliewer, S.A., 2000. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J. Biol. Chem. 275, 15122–15127.

Needham, J., Lu, G., 1970. Proto-endocrinology in medieval China. In: Clerks and Craftsmen in China and the West. Cambridge University Press, pp. 294–315.

Nelson, K.G., Takahashi, T., Bossert, N.L., Walmer, D.K., McLachlan, J.A., 1991. Epidermal growth factor replaces oestrogen in the stimulation of female genital-tract growth and differentiation. Proc. Natl. Acad. Sci. U.S.A. 88, 21–25.

Nelson, L., 2001. Estrogen production in action. J. Am. Acad. Dermatol. 45, S116–S124.

Nishi, M., Takenaka, N., Morita, N., Ito, T., Ozawa, H., Kawata, M., 1999. Real-time imaging of glucocorticoid receptor dynamics in living neurons and glial cells in comparison with non-neural cells. Eur. J. Neurosci. 11, 1927–1936.

Noteboom, W.D., Gorski, J., 1965. Stereospecific binding of estrogens in the rate uterus. Arch. Biochem. Biophys. 111, 559–568.

Obr, A.E., Edwards, D.P., 2012. The biology of progesterone receptor in the normal mammary gland and in breast cancer. Mol. Cell Endocrinol. 357, 4–17.

Ogawa, S., Mozach, J., Benner, C., Tangirala, R.K., Westin, S., Hoffmann, A., Subramaniam, S., David, M., Rosenfeld, M.G., Glass, C.K., 2005. Molecular determinants of crosstalk between nuclear receptors and Toll-like receptors. Cell 122, 707–721.

Orlov, I., Rochel, N., Moras, D., Klaholz, B.P., 2012. Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. EMBO J. 31, 291–300.

Osborne, C.S., 2004. Active genes dynamically colocalize to shared sites of ongoing transcription. Nat. Genet. 36, 1071–1965.

Paul, S.M., Purdy, R.H., 1992. Neuroactive steroids. FASEB J. 6, 2311–2322.

Pedram, A., Razandi, M., Levin, E.R., 2006. Nature of functional estrogen receptors at the plasma membrane. Mol. Endocrinol. 20, 1996–2009.

Pelling, C., 1959. Chromosomal synthesis of ribonucleic acid as shown by the incorporation of uridine labelled with tritium. Nature 184, 655–656.

Perissi, V., Rosenfeld, M.G., 2005. Controlling nuclear receptors: the circular logic of cofactor cycles. Nat. Rev. Mol. Cell Biol. 6, 542–554.

Phelps, C., Gburcik, V., Suslova, E., et al., 2006. Fungi and animals may share a common ancestor to nuclear receptors. Proc. Natl. Acad. Sci U.S.A. 103, 7077–7081.

Pietras, R.J., Szego, C.M., 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265, 69–72.

Pratt, W.B., Galigniana, M.D., Harrell, J.M., DeFRanco, D.B., 2004. Role of hsp90 and the hsp90-binding immunophillins in signalling protein movement. Cell Signal. 16, 857–872.

Ratman, D., vanden Berge, W., Dejager, L., Libert, C., Tavernier, J., Beck, I.M., de Bosscher, K., 2013. How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. Mol. Cell Endocrinol. 380, 41–54.

Razandi, M., Pedram, A., Merchenthaler, I., Green, G.L., Levin, E.R., 2004. Plasma membrane estrogen receptors exist and functions as dimers. Mol. Endocrinol. 18, 2854–2865.

Reddy, J.K., Rao, M.S., 1986. Peroxisome proliferators and cancer: mechanisms and implications. Trends Pharmacol. Sci. 7, 438–443.

Ren, D., Navarro, B., Jackson, A.C., Hsu, S., Shi, Q., Tilly, J.L., Clapham, D.E., 2001. A sperm ion channel required for sperm motility and male fertility. Nature 413, 603–609.

Rezg, R., Salloua, E.-F., Najoua, G., Bessem, M., 2014. Bisphennol A and human chronic diseases. Current evidences, possible mechanisms and future perspectives. Environ. Intern. 64, 83–90.

Rios, A., Fu, N.Y., Lindeman, G.J., Visvader, J.E., 2014. In situ indentification of bipotent stem cells in the mammary gland. Nature 506, 322–327.

Ritossa, F., 1962. A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia 18, 571–573.

Robinson-Rechavi, M., Laudet, V., 2001. How many nuclear hormone receptors in the human genome? Trends Genet. 17, 554–556.

Rochel, N., Ciesielski, F., Godet, J., Moman, E., Roessle, M., Peluso-Iltis, C., Moulin, M., Haertlein, M., Callow, P., Mely, Y., Svergun, D.I., Moras, D., 2011. Common architecture of nuclear receptor heterodimers on DNA direct repeat elements with different spacing. Nat. Struct. Mol. Biol. 18, 564–570.

Rochette-Egly, C., 2003. Nuclear receptors: integration of multiple signalling pathways through phosphorylation. Cell Signal. 15, 355–366.

Rogatsky, I., Luecke, H.F., Leitman, D.C., Leitman, D.C., Yamamoto, K.R., 2002. Alternative surfaces off transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. Proc. Natl. Acad. Sci. U.S.A. 99, 16701–16706.

Rohl, A., Rohrberg, J., Buchner, J., 2013. The chaperone Hsp90: changing partners for demanding clients. Trends Biochem. Sci. 39, 253–262.

Rokaw, C.J., Josslin, R., Bobrow, L., 1974. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. Biochim. Biophys. Res Commun. 58, 795–800.

Rousseau, G.G., Baxter, J.D., Higgins, S.J., Tomkins, G.M., 1973. Steroid induced nuclear binding of glucocortoid receptors in intact hepatoma cells. J. Mol. Biol. 79, 539–554.

Saucedo-Cardenas, O., Quintana-Hau, J.D., Le, W.-D., Smidt, M.P., Cox, J.J., De Mayo, F., Burbach, P.H., Conneely, O.M., 1998. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc. Natl. Acad. Sci. U.S.A. 95, 4013–4018.

Selye, H., 1942. Correlations between the chemical structure and the pharmacological actions of the steroids. Endocrinology 30, 437–453.

Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.-L., Wu, L., Lindeman, G.J., Visvader, J.E., 2006. Generation of a functional mammary gland from a single stem cell. Nature 439, 84–88.

Simpson, S.A., Tait, J.F., Wettstein, A., Neher, R., Euw, J.V., Schindler, O., Reichstein, T., 1954. Aldosteronisolierung und Eigenschaften über Bestandteile de Nebennierenrinde und verwandte Stoffe. Helv. Chim. Acta 37, 1163–1200.

Slotta, K.H., Ruschig, H., Fels, E., 1934. Reindarstellung der hormone aus dem corpus luteum. Krystallographische Untersuchungen von A Neuhaus. Ber. Dtsch. Chem. Ges. 67, 1270–1273.

Smith, E.F., 2002. Regulation of flagellar dynein by calcium and a role for an axonemal calmodulin and calmodulin-dpendent kinase. Mol. Biol. Cell 13, 3303–3313.

Soma, K.K., 2006. Testosterone and aggression: Berthold, birds and beyond. J. Neuroendocrinol. 18, 543–551.

Spach, C., Streeten, D.H., 1964. Retardation of sodium exchange in dog erythrocytes by physiological concentrations of aldosterone, in vitro. J. Clin. Invest. 43, 217–227.

Spiegel, F., 1996. Sick Notes: An Alphabetical Browsing Book of Derivations, Abbreviations, Mnemonics and Slang for the Amusement and Edification of Medics, Nurses, Patients and Hypochondriacs. The Parthenon Publishing Group Ltd, Carnforth, Lancs., UK.

Stenzel, D., Wilsch-Brauninger, M., Wong, F.K., Heurer, H., Huttner, W.B., 2014. Integrin avb3 and thyroid hormones promote expansion of progenitors in embryonic neocortex. Development 141, 795–806.

Stingl, J., Eavec, C.J., Kuusk, U., Emerman, J.T., 1998. Phenotypic and functional characterization in vivo of a multipotent epithelial cell present in the normal human breast. Differentation 63, 201–213.

Stommel, E.W., Stephens, R.E., Masure, H.R., Head, J.F., 1982. Specific localization of scallop gill epithelial calmodulin in cilia. J. Cell Biol. 92, 622–628.

Strunker, T., Goowin, N., Brenker, C., Kashikar, N.D., Weyand, I., Seifert, R., Kaupp, U.B., 2011. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature 471, 382–386.

Surjit, M., Ganti, K.P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M., Chambon, P., 2011. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. Cell 145, 224–241.

Takahashi, T., Kimoto, T., Tanabe, N., Hattori, T.-A., Yasumatsu, N., Kawato, S., 2002. Corticosterone acutely prolonged N-methyl-D-aspartate receptor-mediated Ca^{2+}-elevation in cultured rat hippocampal neurons. J. Neurochem. 83, 1441–1451.

Tan, W., Zhang, W., Strasner, A., Givennikov, S., Cheng, J.Q., Hoffman, R.M., Karin, M., 2011. Tumour-infiltrating regulatory T-cells stimulate mammary cancer metastasis through RANKL-RANK signalling. Nature 470, 548–553.

Tao, Y., Williams-Skipp, C., Scheinman, R.I., 2001. Mapping of glucocorticoid receptor DNA binding domain surfaces contributing to transrepression of NF kappa B and induction of apoptosis. J. Biol. Chem. 276, 2329–2332.

Tissieres, A., Mitchell, H.K., Tracy, U.M., 1974. Protein synthesis in salivary glands of *Drosophila mellanogaster*: relation to chromosome puff. J. Mol. Biol. 84, 389–398.

Toft, D.O., Gorski, J., 1966. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc. Natl. Acad. Sci. U.S.A. 55, 1574–1581.

Traumer, M., Wagner, M., Fickert, P., Zollner, G., 2005. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. J. Clin. Gastroenterol. 39, S111–S124.

Tyagi, S., Gupta, P., Saini, A.S., Kaushal, C., Sharma, S., 2011. The peroxisome proliferator-activated receptor: a family of nuclear receptors role in various diseases. J. Adv. Pharm. Technol. Res. 2, 236–240.

Vandenberg, L.N., Maffini, M.V., Wadia, P.R., Sonnenschein, C., Rubin, B.S., Soto, A.M., 2007. Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alter development of the foetal mouse mammary gland. Endocrinology 148, 116–127.

Vo, N., Goodman, R.H., 2001. CREB-binding protein and p300 in transcriptional regulation. J. Biol. Chem. 276, 13505–13508.

Wang, Z., Benoit, G., Liu, J., et al., 2003. Structure and function of Nurr1 idetifies a class of ligand-independent nuclear receptors. Nature 423, 555–556.

Wang, Y., Chirgadze, N.Y., Briggs, S.L., Khan, S., Jensen, E.V., Burris, T.P., 2006. A second binding site for hydrotamoxifen within the coactivator-binding groove of estrogen receptor beta. Proc. Natl. Acad. Sci. 103, 9908–9911.

Wehling, M., 1995. Aldosterone specific membrane receptors, rapid activation of the sodium-hydrogen exchanger, and cardiovascular implications. Cardiovasc. Res. 29, 167–171.

Whitlock, J.R., Heynen, A.J., Shuler, M.G., Bear, M.F., 2006. Learning induces long-term potentiation in the hippocampus. Science 313, 1093–1097.

Wintersteiner, O., Allen, W.M., 1934. Crystalline progestin. J. Biol. Chem. 107, 321–336.

Wisely, G.B., Miller, A.B., Davis, R.G., Thornquest, A.D., Johnson, R., Spitzer, T., Sefler, A., Shearer, B., Moore, J.T., Miller, A.B., Willson, T.M., Williams, S.P., et al., 2002. Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. Structure 10, 1225–1234.

Yang, P., Diener, D.R., Rosenbaum, J.L., Sale, W.S., 2001. Localization of calmodulin and dynein light chain LC8 in flagellar radial spokes. J. Cell Biol. 153, 1315–1326.

Yoneda, T., Tanaka, S., Hata, K., 2013. Role of RANKL/RANK in primary and secondary breast cancer. World J. Orthop. 4, 178–185.

Zhimulef, I.F., Vokovoa, E.I., Belyaeva, E.S., 2001. Underdevelopment of late prepupal puffs in mutant l(3)63Ea in *Drosophila melanogaster*. Dros. Inf. Serv. 84, 1–4.

Zhou, W., Slingerland, J.M., 2014. Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. Nat. Rev. Cancer 14, 26–38.

Zondek, B., 1928. Das hormon des hypophysenvorderlappens. Klin. Wochenschr. 7, 831–835.

van der Zand, Tabak, H.F., 2013. Peroxisomess: offshoots of the ER. Curr. Opin. Cell Biol. 25, 449–454.

CHAPTER

9

Protein Kinase C in Oncogenic Transformation and Cell Polarity

Protein kinase C (PRKC or PKC) is the third founder member of the group of AGC kinases. As you will learn below, the kinase initially attracted great attention because of its activation by the tumor-promoter phorbol ester. However, it proved very difficult to provide a genuine oncogenic role; and with the discovery of numerous oncogenes that operate in growth factor signaling pathways, such as ERBB, SRC, RAS, or RAF attention shifted to the study of more productive lines of research. As a consequence, protein kinase C obtained the role as a modulator of principal signaling pathways. For instance, we have shown in Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," how protein kinase contributes to the sensitization of adrenalin-mediated airway smooth muscle contraction and in Chapter 7, "Bringing the Signal to the Nucleus," how it contributes to the insulin-mediated arrest of the gluconeogenic program. In this chapter we will elaborate on the composition of the protein kinase C family, on structural details of the catalytic domains, and on the different modes of activation. We will then place protein kinase C in the context of oncogenic cell transformation (tumor promotion) and in the process of spindle orientation during cell division.

AGC PROTEIN KINASES IN OTHER CHAPTERS

Of the members of the AGC protein-kinases group, we have dealt with protein kinase A in Chapter 2, "An Introduction to Signal Transduction" and in Chapter 7, "Bringing the Signal into the Nucleus: Regulation of Gene Expression." We have dealt with protein kinase G in Chapter 4, "Cholinergic Signaling and Muscle Contraction." In Chapter 16, "Signaling through the Insulin Receptor," we discuss yet another member, protein kinase B (AKT).

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DISCOVERY OF A PHOSPHORYLATING ACTIVITY INDEPENDENT OF cAMP

The enzymes that we call protein kinase C became apparent during the late 1970s as a cyclic nucleotide-independent protein kinase present in bovine cerebellum (Takai et al., 1977). As first described, this activity appeared to be the product of limited proteolysis by a calcium-dependent protease (Inoue et al., 1977) but shortly afterward, it was found to be activated by Ca^{2+} (50 μmol/l) and phospholipids, with no need for proteolysis (Takai et al., 1979). More significantly, the activity of this protein kinase could also be stimulated by the addition of a small quantity of diacylglycerol (DAG), product of the phospholipase-C reaction, together with phospholipids (generally phosphatidylserine) and Ca^{2+}, now at concentrations not far above the physiological range (2–6 μmol/l) (Kishimoto et al., 1980).

The new enzyme became a focus of interest when it was found that it constitutes the predominant intracellular target for the active principles (phorbol esters) of croton oil, long recognized as skin irritants having tumor-promoting capacities (van Duuren et al., 1963; Castagna et al., 1982) (Figure 9-1). Such phorbol esters activate protein kinase C, substituting for, and competing with, the physiological diacylglycerol (Crombie et al., 1968; Sharkey et al., 1984). Phorbol ester has since been applied to every cell and system imaginable. It is apparent that protein kinase C is involved in an enormous number of cellular processes. These range from tumor formation, host defense, embryological development, pain perception, neurite outgrowth, and the development of long-term memory.

The term phorbol ester is commonly used as a sort of slang stand-in for the compound phorbol 12-myristate 13-acetate or 12-*O*-tetradecanoyl phorbol-13-acetate. The alternative abbreviations PMA or TPA, respectively, appear widely in the literature. In general, we have preferred the term PMA but TPA cannot be avoided as it gives rise to the TRE, the TPA-response element.

Croton oil, a poisonous viscous liquid obtained from the seeds of a small Asiatic tree, Croton tiglium, a member of the spurge family (Euphorbiaceae). The tree is native to India and the Indonesian Archipelago. Croton oil is pale yellow to brown and is transparent, with an acrid persistent taste and disagreeable odor. It is a violent irritant. The use of croton oil as a drug was introduced to the West from China by the Dutch in the sixteenth century. The nineteenth century physician (and pioneer in the field of Signal Transduction) Sidney Ringer describes its use as a purgative and its topical application in the treatment of ringworm (Ringer, 1888). It is now considered too dangerous for medicinal use. For more information about Sidney Ringer, return to Chapter 6 "Intracellular Calcium," section "A new second messenger is discovered."

The symptoms of inflammation were summarized for all time by the first century Roman encyclopedist, Celcius, as tumor, calor, rubor,

continued

and dolor (swelling, warmth, redness, and pain, respectively). Inflammation, now recognized as a type of nonspecific immune response, represents the basic means by which the body reacts to infection, irritation, or injury.

A citation-classic review about protein kinase C: (Nishizuka (1984)).

A review about how the TRE, downstream response-element of TPA, is involved in skin inflammation: (Zenz et al., 2008).

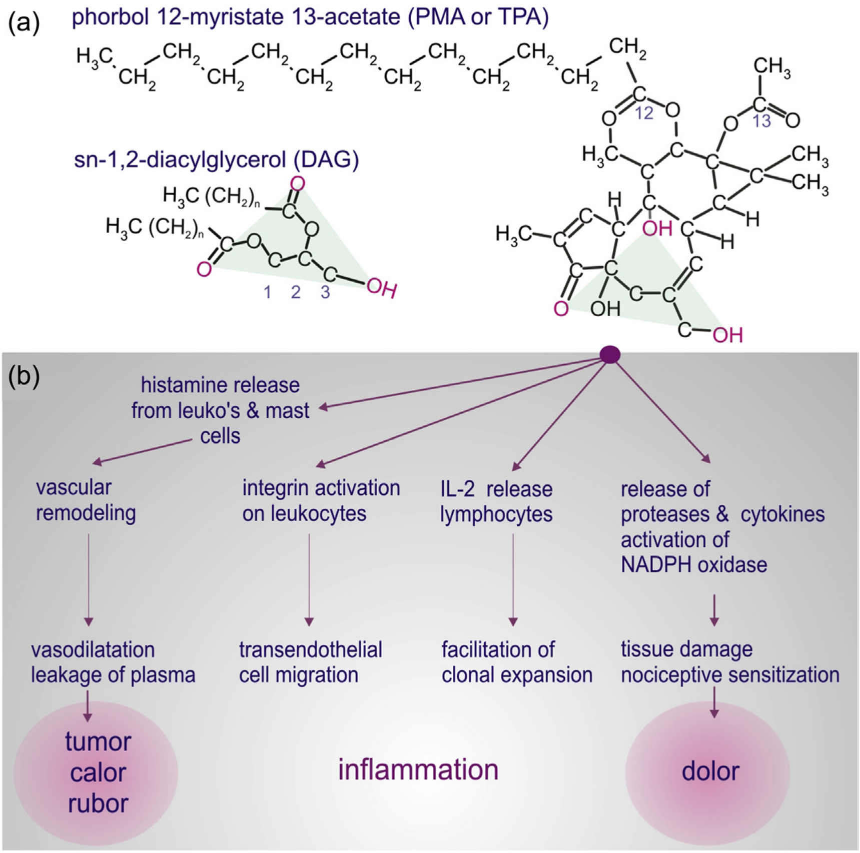

(a)
phorbol 12-myristate 13-acetate (PMA or TPA)
sn-1,2-diacylglycerol (DAG)
1 2 3
12
13
(b)
histamine release from leuko's & mast cells
vascular remodeling
integrin activation on leukocytes
IL-2 release lymphocytes
release of proteases & cytokines activation of NADPH oxidase
vasodilatation leakage of plasma
transendothelial cell migration
facilitation of clonal expansion
tissue damage nociceptive sensitization
tumor
calor
rubor
inflammation
dolor

FIGURE 9-1 Phorbol ester and the induction of inflammation. (a) Diacylglycerol and the phorbol 12-myristate 13-acetate are unrelated structures except for the similar orientation of three critical oxygens (in pink). These are essential for activation of protein kinase C and constitute the "pharmocophore." (b) Dermal application of phorbol ester causes inflammation (acting as an irritant) as witnessed by tumor (swelling), calor (warmness), rubor (redness), and dolor (pain). The symptoms are the consequences of its stimulation of histamine release, activation of integrins, stimulation of lymphocytes (IL-2-mediated expansion), and the release of proteases, cytokines, and reactive oxygen and nitrogen species by neutrophils and macrophages (Janoff et al., 2000; Kavanaugh et al., 1991; Rosenstreich and Mizel, 1979; Sun et al., 2000; Repine et al., 1974; Katakami et al., 1984).

THE PROTEIN KINASE C FAMILY

Molecular cloning reveals a family of 12 genes giving rise to 13 distinct enzymes (Parker et al., 1986; Coussens et al., 1986; Palmer et al., 1994). Not all of these are activated by phorbol esters (Nishizuka, 1995). They can be subdivided into three subfamilies (Figure 9-2)—conventional, novel, and atypical protein kinases. The atypical really differ with respect to their

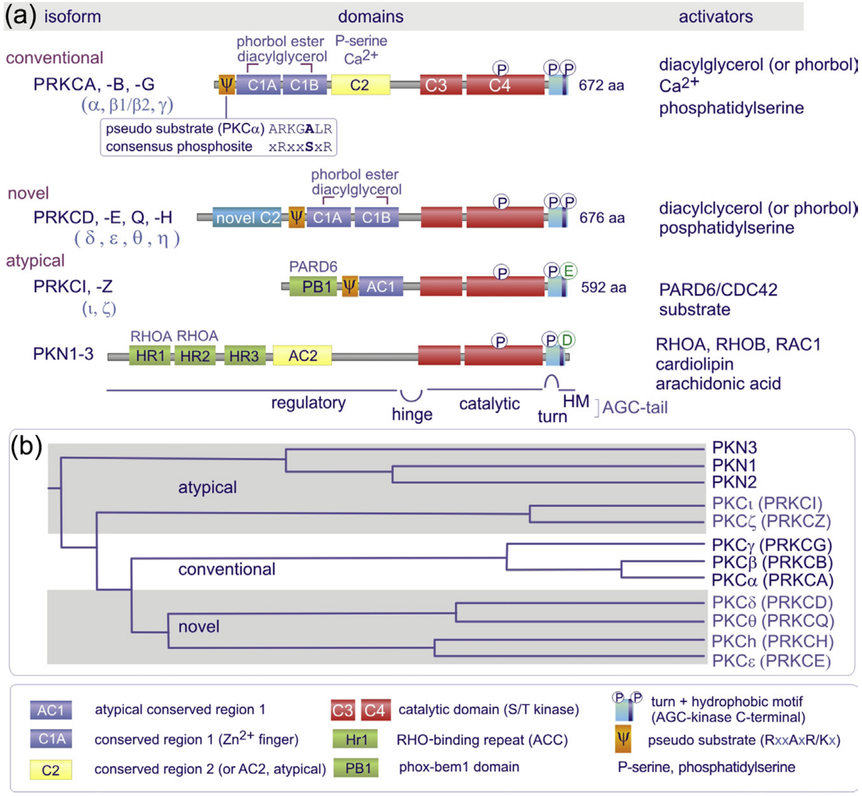


FIGURE 9-2 The family of protein kinases C. (a) Mammalian protein kinase C comprises a family of 12 distinct members, giving rise to 13 isoforms, subdivided into three subfamilies—four conventional (α, β1, β2, and γ), four novel (δ, ε, θ, and, η), and five atypical (ι, ζ, and PKN1–3). They are identified as protein kinase C by the conserved domains C1, C2, C3, C4, and the characteristic AGC-kinase C-terminal. The proteins are made up of regulatory and catalytic domains, connected by a hinge region. PKCβ1 and –β2 are splice variants of the same gene. An alternative transcript exists for PKCζ which only encodes the catalytic domain and is referred to as PKMζ (not shown). Note the different modes of activation of the protein kinases, which varies from Ca^{2+}, lipids to monomeric GTP-binding proteins (CDC42, RAC1, or RHOA). (b) The sequence alignment shows the relatedness of the different protein kinase C isoforms. Notice that although they are arranged among the atypical group, the three PKN members are a separate branch that had split off early in evolution.

mode of activation. Here we will not consider the PKN members. The various isoforms are also referred to as "isozymes" but this term is better reserved for an enzyme such as lactate dehydrogenase, since the isoforms of PKC have separate and characteristic substrates.

The three subgroups of protein kinase C are recognized on the basis of sequence similarities, particularly in the catalytic domains, the characteristic AGC terminal, and their modes of activation. The conventional protein kinases C are all activated by diacylglycerol and Ca^{2+} against a background of phosphatidylserine. The novel isoforms require phospholipid and diacylglycerol, but not Ca^{2+}. This is explained by the presence of an alternative C2 domain, which does not bind Ca^{2+}. The atypical isoforms respond neither to diacylglycerol nor Ca^{2+} and seem not to require phospholipids. They carry an atypical C1 or C2 domain and have additional domains such as PB1 (protein–protein interaction domain) or homology regions 1, 2, and 3 (HR1–3, also known as antiparallel coiled-coil finger). They are activated by monomeric GTPases such as CDC42 and members of RHO (Joberty et al., 2000; Amano et al., 1996). All the PKCs share a common serine/threonine kinase catalytic domain comprising two highly conserved subdomains, C3 (N-lobe) and C4 (C-lobe), and they possess an AGC-kinase terminal domain which is characterized by a turn and hydrophobic motifs (with highly conserved phenylalanines).

Phosphorylation of substrate by PKC occurs only at serine and threonine residues in the close vicinity of arginine residues situated in the consensus sequence, RxxS/TxRx (Figure 9-6). This is present in many proteins and just for PKCα some 800 substrates have been identified (for a detailed discussion, see Nishikawa et al., 1997). As a result, the protein kinases C can be regarded as a family of broad-specificity kinases, having differences in substrate recognition between the subfamilies. Thus, all forms of protein kinase C (with the exception of PKCζ) are capable of phosphorylating MARCKS and GAP-43 while ribonucleoprotein A1 (hnR A1) is only efficiently phosphorylated by PKCζ. Additionally, different levels of expression of the various protein kinase isoforms and scaffold proteins in tissues may contribute to particular tissue- or cell-specific responses to hormones, growth factors, cytokines, or neurotransmitters (Ohno et al., 1987). While some protein kinase C isoforms are expressed in a tissue-specific manner (i.e., PKCθ is expressed primarily by skeletal muscle, lymphoid organs, and hematopoietic cell lines, and PKCγ is detected large in neuronal tissues), most isoforms are ubiquitous and many cells co-express multiple family members (Steinberg, 2008).

Genes coding for pkc-2 (conventional), pkc-1 and tpa-1 (novel), pkc-3 (atypical), and Pkn-1 (atypical) are present in *Caenorhabditis elegans* (Tabuse, 2002) and four isotypes, dPKC1, -3, InaC, and a putative dPKCδ, are present in *Drosophila* (Rosentahl et al., 1987). Genetic screening of these organisms has provided insights into the functioning of protein kinase C, in particular with respect to the role of assembling proteins in the formation of large signaling complexes (see below) and cell polarity.

For substrate phosphosite sequence logos of the different members of the protein kinase C family we refer to PhosphoSitePlus (type gene symbols as provided in our figures: PRKCA, PRKCD, PKN1, etc.): www.phosphosite.org.

STRUCTURAL COMPOSITION OF PROTEIN KINASE C

The deduced amino acid sequences reveal four reasonably conserved domains, C1–C4, having similarities to those present in other signaling proteins (Newton, 1995) (Figures 9-2 and 9-3). Proceeding from the N-terminus, C1 and C2 constitute the regulatory domains and then C3 and C4 together constitute the catalytic domain characteristic of all kinases (return to Chapter 2, "An Introduction to Signal Transduction," Figure 2-35).

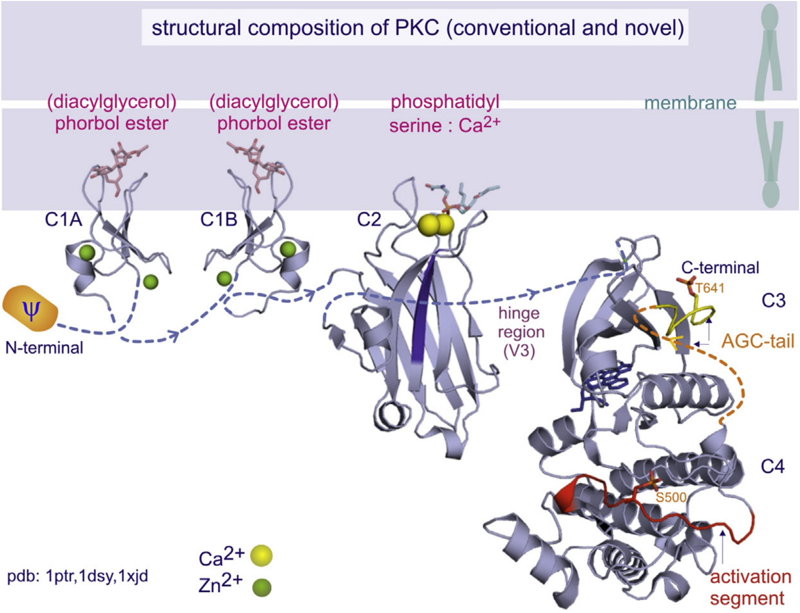

structural composition of PKC (conventional and novel)
(diacylglycerol) phorbol ester
(diacylglycerol) phorbol ester
phosphatidyl serine : Ca2+
membrane
C1A
C1B
C2
ψ
N-terminal
hinge region (V3)
C-terminal
T641
C3
AGC-tail
C4
S500
activation segment
pdb: 1ptr,1dsy,1xjd
Ca2+
Zn2+

FIGURE 9-3 Structural composition of PKC. The conserved domains C1, 2, 3, and 4 are functional modules. C1 (A+B) binds diacylglycerol or phorbol ester, C2 is involved in the attachment to phospholipid, which is reinforced by the binding of Ca^{2+}, and C3+C4 constitute the catalytic entity which is linked to C2 by a hinge region (V3). Regulation of activity and interaction with the upstream kinase PDK1 occurs through the turn and hydrophobic (HM) motifs (AGC-tail). The structures shown are a compilation of conventional and novel PKC isoforms: C1 (A+B) domain of PKCδ, a C2 domain of PKCα and C3/C4 domains of PKCθ.

The C1 domain

The C1 domain contains a zinc finger motif that forms the binding site for diacylglycerol and phorbol esters in the context of phospholipids. The stimulus-mediated generation of diacylglycerol effectively plugs a hydrophilic site in the C1 domain, making the surface more hydrophobic, so allowing C1 to become buried in the membrane (Zhang et al., 1995). The C1 domain of the novel PKCs has an enhanced affinity for diacylglycerol whereas the atypical PKCs (ι, ζ) have an impaired C1 domain and respond to neither diacylgycerol nor phorbol ester. Importantly, in the absence of diacylglycerol, the C1B domain makes contacts with the catalytic domain at the level of a conserved NFD motif (asparagine–phenylalanine–aspartate) of the AGC tail. Within a predominantly hydrophobic environment the adjacent proline and alanine residues provide hydrogen bonds that serve as a counterpart of the hydroxyl groups of diacylglycerol or phorbol ester (return to Figure 9-1). The contacts between C1B and the catalytic domain mimic membrane and second messenger. As a consequence of the C1B clamp, phenylalanine (position 629 in rat PKCβII), which normally binds to the adenine of ATP, turns more outward and this abrogates the correct coordination of ATP (see Figure 9-5) (Leonard et al., 2011). We note that the C1B domain contains a nuclear- (NLS) and Golgi-localization signal sequence (Kheifets and Mochly-Rosen, 2007). We also note that in addition to members of the PKC family, other proteins contain a typical C1 domain and they too are regulated by diacylglycerol or phorbol esters (textbox) (Hurley et al., 1997).

C1 domains are present in a wide range of other proteins, some of which bind phorbol esters (so-called typical) while others do not (atypical). Examples of phorbol ester-binding proteins that lack kinase activity include the chimaerins (a family of Rac-GTPase-activating proteins), CalDAG–GEF (a Ras/Rap1 guanine-exchange factor), diglyceride kinases, protein kinase D, and Unc-13/Munc-13 (involved in exocytosis) (for review, see Colon-Gonzales and Kazanietz, 2006).

C1 domain as a protein–protein interaction domain

Recent data suggest nonequivalent roles for the C1A and C1B domains in targeting to intracellular compartments such as the Golgi apparatus, mitochondria, and the nucleus. The C1B domain of PKCα interacts with fascin, a cell matrix protein, and this may have an inhibitory effect on cell migration. The C1B domain of PKCγ binds 14-3-3τ, important in the regulation of solute transport across Gap junctions. The C1A of PKCβII binds to pericentrin, a scaffold protein of the centrosome. Loss of this interaction prevents mitotic spindle formation, and since this structure is necessary for the segregation of chromosomes, it prevents mitosis (Colon-Gonzales and Kazanietz, 2006).

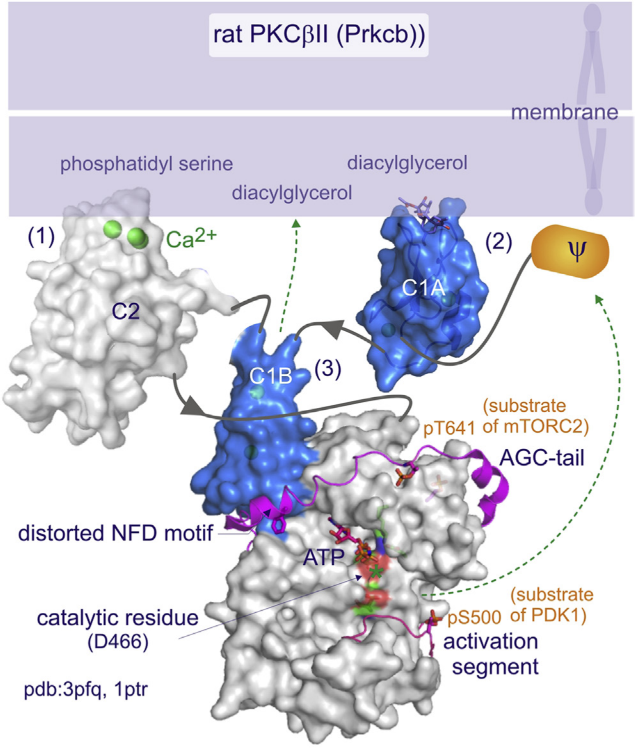


FIGURE 9-4 Role of C1B in holding protein kinase C in an inactive state. The figure represents a model of conventional protein kinase-βII (crystallized) in an intermediate activation state. The C2 domain is buried in the membrane due to the presence of three calcium ions. The C1A domain is also buried in the membrane, attached to diacylglycerol (or phorbol ester) and as a consequence the pseudosubstrate is removed from the catalytic cleft. Although substrate may now have access, the kinase remains inactive because the remaining C1B domain clamps onto the NFD motif in the AGC tail and thus distorts the position of a phenylalanine (F629). As a result ATP is not properly coordinated and phosphate transfer cannot occur. Only when the C1B domain translocates to the membrane can we speak of a fully active protein kinase (Leonard et al., 2011).

The C2 domain

The C2 domain binds, in the presence of two calcium ions, to negatively charged phospholipid head groups, such as that of phosphatidylserine. The C2 domain of novel PKC (“novel C2”) lacks key residues involved in Ca^{2+} binding, and as a consequence it binds neither Ca^{2+} nor phospholipids (Verdaguer et al., 1999). Surprisingly, in the case of PKCδ and -θ, the C2 domain binds phosphotyrosine residues instead (so providing an opportunity to link tyrosine receptor kinase pathways with those of PKC) (Benes et al., 2005). C2 domains are also present in a number of other signaling molecules, among which, phospholipases, GTPase-activating proteins and perforin (a membrane pore–forming protein released by cytotoxic immune cells). We note that the C2 domain contains a RACK1 (see below) and a nuclear lamin-A-binding motif (Kheifets and Mochly-Rosen, 2007).

The C3 and C4 domain

The C3 and C4 domains together constitute a serine/threonine kinase domain with a characteristic ATP-binding N-lobe and a C-lobe comprising an activation segment that must be phosphorylated to achieve catalytic competence (with perhaps an exception for PKCι and -ζ). Like for all other AGC-protein kinases the C lobe harbors the catalytic residue, an aspartate, occurring in the highly conserved RDL motif (return to Chapter 2, "An Introduction to Signal Transduction," Figure 2-35).

The AGC-kinase C-terminal sequence (AGC tail), comprising a turn and hydrophobic motifs, plays an important regulatory role. It is involved in the binding and the activation of the upstream PDK1 and it controls the conformation of the N-lobe and therefore the precise positioning of ATP. There are two essential phosphorylation sites in the AGC tail, one of which places the hydrophobic motif against the N-lobe. In the atypical protein kinases the serine in the hydrophobic motif is replaced by a glutamate or aspartate (Figure 9-2). We note that, apart from the above-described intramolecular interactions the AGC tail also interacts with RACK1, PDZ domains and contains a nuclear localization signal (NLS) (Kheifets and Mochly-Rosen, 2007).

Pseudosubstrate (ψ) and C1B domain

Protein kinase C is maintained folded and inactive by (at least) two intramolecular interactions. One involves the segment immediately N-terminal to the C1 domain (residues 19–36 in PKCα) that constitutes the autoinhibitory pseudosubstrate (ψ). The sequence resembles the consensus phosphorylation sites present in target proteins that are phosphorylated by PKC (example shown in Figure 9-2). However, the pseudosubstrate possesses an alanine at the position occupied by serine in the substrates and is consequently not amenable to phosphorylation (House and Kemp, 1987). In the absence of a stimulus, the catalytic domain binds to the pseudosubstrate, causing the enzyme to fold about the hinge. This brings about a second intramolecular interaction by placing the C1B domain against the N-lobe and the AGC tail (at the level of the NFD motif), thus distorting the coordination of ATP (Leonard et al., 2011). As a consequence, kinase activity is suppressed.

PB1 domain

The PB1 domain, initially found in the phagocyte NADP oxidase proteins p67Phox and in the yeast polarity protein Bem 1, is a protein–protein interaction domain. The PB1 domain comprises 80 amino acids and are grouped into three types: Type I, contains a conserved acidic Dx(D/E)GD segment that goes under the name OPCA motif (for OPR/PC/AID motif) and interacts with conserved lysine residues from a type II domain. Proteins with a type-I domain are CDC42, p40Phox (NCF4), MEK5 (MAP2K5), and NBR1. Type II, contains a conserved lysine residue and occurs in NCF2 (p67Phox), PAR-6 (PADR6), MEKK2 (MAP3K2), and MEKK3 (MAP3K3). Type I/II, contains both OPCA and a conserved lysine and thus possesses two interaction surfaces. This type is found in atypical protein kinase-ι and ζ (PRKCI and PRKCZ), p62 or sequestosome-1 (SQSTM1), and in TGF (Figure 9-5). Type 1 and type II domains interact with each other in a front-to-back fashion, resulting in heterodimers in which acidic residues of the OPCA motif form electrostatic contacts with the basic histidine of the type II domain. The type I/II domain allows for homotypic front-to-back arrays (oligomers) (Wilson et al., 2003; Moscat et al., 2006).

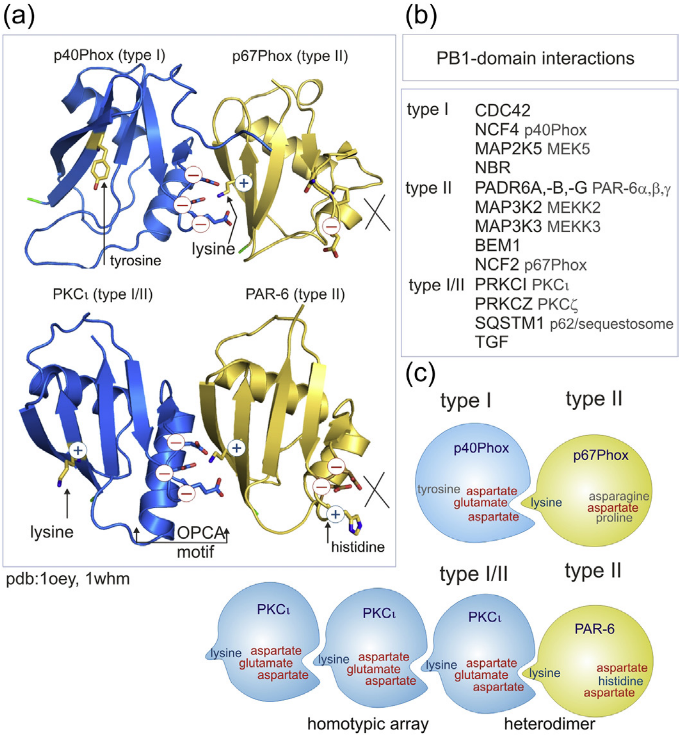


FIGURE 9-5 PB1 structures and domain interactions. (a) Structures of type I, type II, and type I/II PB1 domains. Type 1 domains carry a cluster of acidic residues (–) in a conserved Dx(D/E) GD segment (also known as OPCA motif). This cluster surrounds the characteristic lysine (+) of the type II domain and forms the backbone of domain interaction. Note that the lysine in the type I domain is replaced by tyrosine and that the type II domain lacks a functional acidic segment. It either contains only one aspartate (–), which is not pointing outward (p67Phox), or it contains two acidic residues but combined with a positively charged histidine (PAR-6). The structure of the type I/II domain (PKCι) carries both the cluster of acidic residues and the basic lysine and can thus form homotypic arrays. (b) List of proteins carrying a type I, type II, or type I/II domain. (c) Schematic representation of the interactions between the different PB1 domains.

PRIMING AND ACTIVATION OF CONVENTIONAL AND NOVEL PROTEIN KINASE C

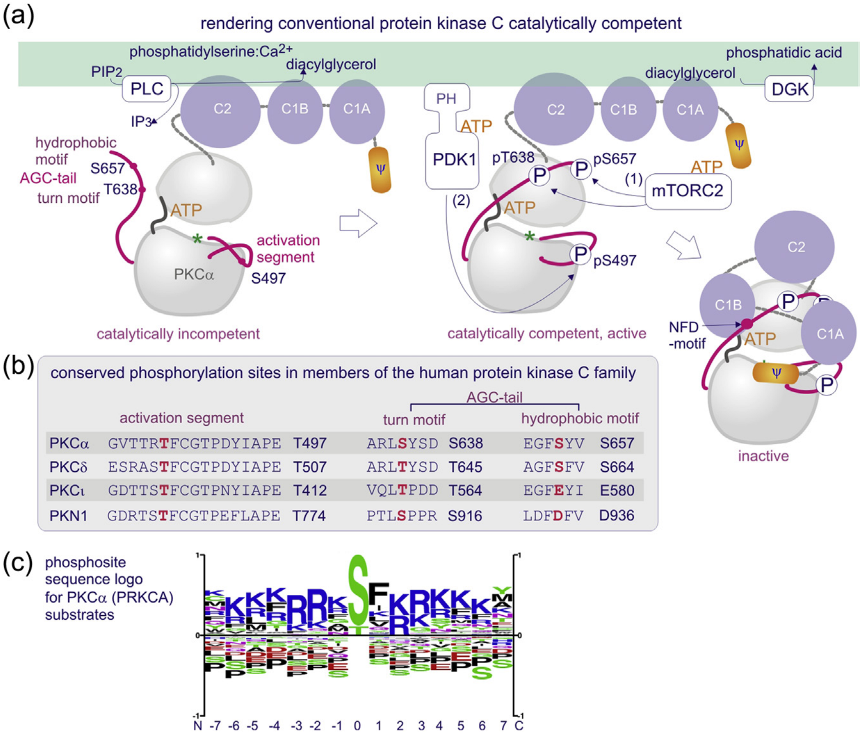
PKC is synthesized as an inactive nonphosphorylated precursor. At this stage the catalytic site is accessible to ATP but the enzyme is catalytically incompetent and requires phosphorylation at two or three residues (process referred to as priming or maturation). Binding of ATP is also essential but not for autophosphorylation purposes because a nonhydrolyzable analog can replace ATP. Unexpectedly, the binding of ATP renders the protein kinase a suitable substrate for its (upstream) activating protein kinases (Cameron et al., 2009). This initial priming step resembles somewhat the switch action of GTP in GTP-binding proteins. Here too binding of the triphosphate nucleotide impacts on interaction with (effector) proteins (return to Chapter 2, "An Introduction to Signal Transduction," Figure 2-25). The first kinase to act on protein kinase C is mTORC2 and it phosphorylates residues in the AGC tail (residues thr-638 and ser-657 for human PKCα). The second, PDK1, phosphorylates the immature kinase in the activation segment (ser-497) (Figure 9-6) (Collins et al., 2005; Facchinetti et al., 2008; Le Good et al., 1998). Phosphorylation of the activation segment takes place at the membrane. The current idea is that membrane association, through binding to diacylglycerol (and Ca^{2+}), both increases the chance of meeting PDK1 and then renders the catalytic domain accessible to its action. In this respect, the role of diacylglycerol seems equivalent, though not identical, to that of phosphatidyl inositol PI-3,4,5-P3 in the activation of yet another member of the AGC family, namely protein kinase B (AKT) (we return in more detail to the action of PDK1 and mTORC in Chapter 16, "Signaling through the Insulin Receptor").

After priming, protein kinase C can be reactivated through the production of diacylglycerol and an increase in the concentration of intracellular free Ca^{2+}. The C2 domain will first bind the membrane in a Ca^{2+}-dependent manner, the C1A domain then interacts with diacylglycerol and in doing so removes the pseudosubstrate from the catalytic cleft (Leonard et al., 2011). Finally, C1B detaches from the N-lobe/AGC tail and joins the C1A domain by binding to diacylglycerol. The kinase is now fully active and it phosphorylates substrates that present serines or threonines within the context of RxxS/TxR (see "phosphosite sequence logo" of protein kinase-C phosphosites) (Figure 9-6).

In test tube experiments, protein kinase C purified from cellular extracts can be activated or deactivated by the addition or removal of phorbol ester (or diacylglycerol) and Ca^{2+}, there is no requirement for mTORC2 or PDK1. Control of activity occurs exclusively at the level of production of the second messengers and not at the level of phosphorylation/dephosphorylation of the protein kinase. Additional control may occur in vivo through the action of protein phosphatases but, like protein kinase A, the mature protein kinase appears to be rather resistant to dephosphorylation (Srivastava et al., 2002; Cameron et al., 2009).

OTHER REGULATORY MECHANISMS OF PROTEIN KINASE C

In addition to phosphorylation, other posttranslational modifications have been detected on protein kinase C. We cite oxidation, acetylation, and nitration. For an extensive review we refer to Steinberg (2008).


(a)
rendering conventional protein kinase C catalytically competent
phosphatidylserine:Ca^{2+}
diacylglycerol
PIP_2
PLC
IP_3
C2
C1B
C1A
ψ
hydrophobic motif
AGC-tail
turn motif
S657
T638
ATP
activation segment
S497
PKCα
catalytically incompetent
PH
ATP
PDK1
(2)
pT638
pS657
P
(1)
ATP
mTORC2
pS497
diacylglycerol
phosphatidic acid
DGK
catalytically competent, active
C2
C1B
NFD -motif
ATP
C1A
ψ
P
inactive
(b)
(c)
phosphosite sequence logo for PKCα (PRKCA) substrates
1
0
-1
N -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 C

conserved phosphorylation sites in members of the human protein kinase C family

	activation segment		turn motif (AGC-tail)		hydrophobic motif (AGC-tail)	
PKCα	GVTTR**T**FCGTPDYIAPE	T497	ARL**S**YSD	S638	EGF**S**YV	S657
PKCδ	ESRAS**T**FCGTPDYIAPE	T507	ARL**T**YSD	T645	AGF**S**FV	S664
PKCι	GDTTS**T**FCGTPNYIAPE	T412	VQL**T**PDD	T564	EGF**E**YI	E580
PKN1	GDRTS**T**FCGTPEFLAPE	T774	PTL**S**PPR	S916	LDF**D**FV	D936

FIGURE 9-6 Priming and activation of protein kinase C. (a) Newly synthesized protein kinase C is processed to render it catalytically competent (referred to as priming or maturation). This involves the binding of ATP to the N-lobe (in the nucleotide-binding pocket) and three subsequent phosphorylations. Two of these occur in the AGC tail by the protein kinase mTORC2 and one occurs in the activation segment by PDK1. The latter event most likely takes place at the membrane following generation of diacylglycerol and the liberation of Ca^{2+}. With depletion of diacylglycerol (conversion to phosphatidate by DGK) and Ca^{2+}, protein kinase C detaches from the membrane and folds onto itself due to binding of its own pseudosubstrate (ψ) and C1B domain. The pseudosubstrate occupies the catalytic cleft and the C1B domain clamps onto the NFD motif in the AGC tail, thus abrogating the proper coordination of ATP. Folded protein kinase C remains competent for some time and re-addition of diacylglycerol and Ca^{2+} suffices to bring it back to life. (b) Priming phosphorylation sites of conventional, novel, and atypical protein kinase C. Note that atypical protein kinase C lack the hydrophobic motif phosphorylation site, they carry a negatively charged glutamate or aspartate instead. (c) "Phosphosite sequence logo" of protein kinase C substrates as revealed by mass spectrometric analysis of 786 input sequences. Positive values indicate the prevalent detection of amino acids within the phosphosite (you nearly always find an R(arg) two amino-acid upstream of the phosphoserine or -threonine), whereas negative values indicate their absence (you would never find an E(glu) one amino-acid upstream). The consensus sequence for protein kinase C is RxxS/TxR but, as the sequence logo shows, some variation exists. *The image is obtained from the PhosphoSitePlus database (Hornbeck et al., 2012).*

PRIMING AND ACTIVATION OF ATYPICAL PROTEIN KINASE C

Things are quite different for the atypical PKCs -ι and -ζ because they do not bind diacylglycerol and they are insensitive to Ca^{2+}. Instead of a C1 and C2 domain they carry a PB1 and an atypical C1 domain. BP1 is a protein–protein interaction domain and has been shown to bind a similar domain present in PAR-6, p62/sequestosome, MEKK2, or MEK5 (Moscat et al., 2006). We will return to PAR-6 in the section about cell polarity. The priming process requires phosphorylation of the AGC tail which contains only one essential phosphorylation site, positioned in the turn motif. The serine normally present in the hydrophobic motif of protein kinase C is replaced by a glutamate (Figure 9-4). Surprisingly, although phosphorylation of the activation segment has been detected, it is not required for full catalytic competence (Ranganathan et al., 2007). The activation process is yet poorly understood. Because of the presence of a pseudosubstrate, the protein is thought to exist in a folded and unfolded conformation (Figure 9-7). Unfolding of the protein may be initiated by certain lipid messengers, such as PI-3,4,5-P3 or ceramide, or the process may be initiated through interaction with proteins such as the monomeric GTPase CDC42 in complex with the polarity protein PAR-6 (Joberty et al., 2000). Unfolding requires both removal of a pseudosubstrate from the catalytic cleft and detachment of the AC1 domain from the N-lobe. This however is not sufficient; binding of substrate is also necessary to bring about yet another conformational change that positions the N-lobe nearer to the C-lobe (closed conformation) and as a result brings the γ-phosphate within reach (<0.3 nm) of the catalytic aspartate (Wang et al., 2012). The enzyme is now ready for phosphate transfer.

MULTIPLE SOURCES OF DIACYLGLYCEROL AND OTHER LIPIDS TO REGULATE PROTEIN KINASE C

In view of the requirement for diacylglycerol, the regulation of members of conventional and novel protein kinase C is clearly linked to the activity of phospholipase C (PLC) (return to Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," section "Phospholipase C," Figures 3-15 and 3-16) (Berridge and Irvine, 2000). The hydrophobicity and small size of diacylglycerol enables it to diffuse laterally in membranes with some rapidity. Virtually all ligands, growth factors, hormones, or neurotransmitters promote the production of diacylglycerol (and IP_3) in one way or another, and this explains why protein kinase C is implicated in a large number of cellular responses. Sustained activation of protein kinase C requires the simultaneous presence of elevated levels of diacylglycerol and high-frequency Ca^{2+} spikes (Oceana and Meyer, 1998). With

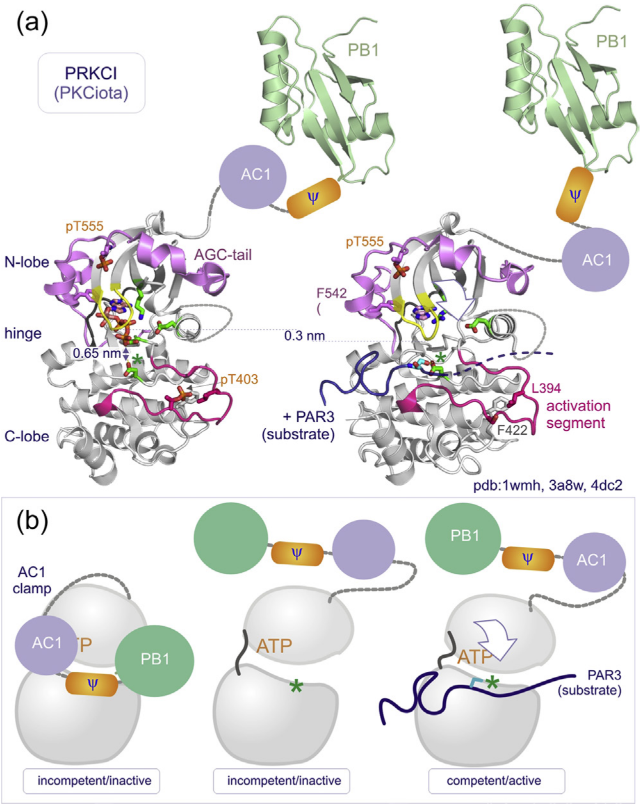


FIGURE 9-7 Priming and activation of the atypical protein kinase Cι (iota). (a) The atypical protein kinase C comprises a PB1 domain, a pseudosubstrate motif, an AC1 domain, and a typical catalytic domain, with an N- and C-lobe embraced by the AGC tail. In the structure on the left, with ATP, both the AGC tail (T555) and the activation segment (T403) are phosphorylated but nevertheless the protein kinase is not operational because the γ-phosphate positions at 0.65nm from the catalytic aspartate (indicated with a green asterisk) which is too far for a nucleophilic attack. The structure on the right carries a nucleotide analog, lacks phosphorylation of the activation segment but is bound to a short peptide, derived from the substrate protein PAR-3. This structure reveals an operational protein kinase, the N-lobe has moved downward toward the C-lobe (indicated by two thin dotted lines) and the substrate is within reach of the catalytic aspartate. Note that despite the lack of phosphorylation of T403, the activation segment displays a correct folding. Stability of the activation segment may be achieved through hydrophobic interactions between L394 and F422 and this explains why phosphorylation is not a prerequisite for kinase activity. (b) Possible model of atypical protein kinase activation. Detachment of the PB1 domain removes the pseudosubstrate from the catalytic cleft and relieves the inhibitory clamp of AC1. Binding of substrate lowers the position of the N-lobe and renders the kinase fully active.

respect to Ca^{2+} spikes, a similar situation has been described for the Ca^{2+}/calmodulin-dependent protein kinase (CaMK), a protein that also oscillates between an autoinhibited compact (folded) and active extended state (unfolded) (return to Chapter 6, “Intracellular Calcium,” Figure 6-18). From this we learn that protein kinase C must oscillate between a

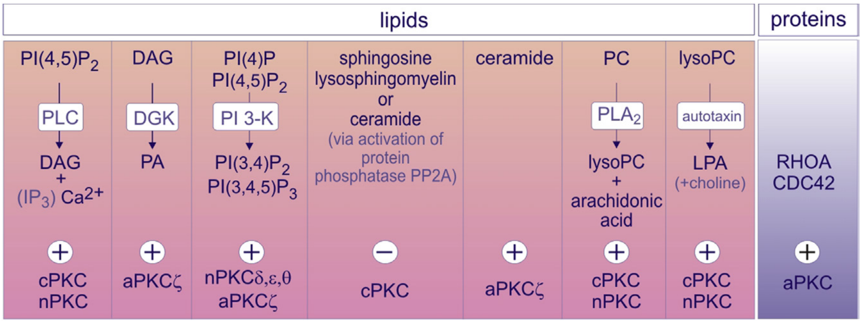


FIGURE 9-8 Multiple lipid sources, and proteins, to activate protein kinase C. Distinct lipid sources and phospholipases and kinases activate the various isoforms of protein kinase C. Uniquely, the atypical PKCι can be activated by the GTPase Cdc42 in the presence of Par-6 and PKNs are targets of RHOA. PLC, phospholipase C; PI(4), phosphatidylinositol-4-phosphate; DAG, diacylglycerol; DGK, diacylglycerol kinase; PA, phosphatidic acid; PLA2, phospholipase A2; a-, c-, nPKC, atypical, conventional, or novel protein kinase C.

folded and unfolded state and that second messengers serve to drive the equilibrium to the open state.

Several other lipid second messengers and mediators either potentiate the effect of diacylglycerol and Ca^{2+} or activate the protein kinase C isoforms directly (Figure 9-8). In particular, the 3-phosphorylated inositol lipids (PI-3,4-P2 and PI-3,4,5-P3, products of the phosphoinositide 3-kinases, see Chapter 16, "Signaling through the Insulin Receptor: Phosphoinositide 3-Kinases and AKT") activate both the novel (δ, ε, θ, and η) and the atypical (ι, ζ) (Toker et al., 1994). Unsaturated fatty acids (particularly arachidonic acid and lysophosphatidic acid) and lyso-phosphatidylcholine also enhance the activity of protein kinase C, potentiating the effects of diacylglycerol at basal Ca^{2+} concentrations (100 nM range) (Sando and Chertihin, 1996). Conversely, the breakdown products of sphingolipids (sphingosine and lysosphingolipids) inhibit conventional protein kinase C members, most likely by masking their interaction with phosphatidyl serine, but activate atypical PKCζ (Hannun and Obeid, 2008). Finally, lipids may have an indirect effect as has been demonstrated for the sphingolipid ceramide. It binds an inhibitor (I2PP2A) of protein phosphatase 2A and this leads to enhanced dephosphorylation of the AGC tail and thus inactivation of PKCα and PKCβII (Kitatani et al., 2007; Mukhopadhyay et al., 2009).

DIFFERENTIAL LOCALIZATION OF PROTEIN KINASE C ISOFORMS

The existence of numerous isoforms, but the lack of individual substrate specificities, raises the question of whether the various protein kinase C isoforms might have redundant roles. Immunocytochemical analysis however reveals that particular isoforms are present in different cells and subcellular compartments and bind to unique protein complexes. They may have specialized functions. The first indication was provided by a study of inductive signaling in *Xenopus laevis* embryos. Here, dorsal ectoderm is more competent to develop as neural tissue than the ventral ectoderm. The difference persists when an artificial stimulus (phorbol ester) is applied. PKCα is preferentially expressed in dorsal ectoderm, whereas PKCβ is uniformly distributed (Otte et al., 1991). Overexpression of PKCα in ventral ectoderm annuls the difference, the ventral ectoderm becoming fully competent to form neural tissue (Otte and Moon, 1992).

Overexpressed isoforms of protein kinase C tend to be diffusely distributed throughout the cytoplasm of fibroblasts, with just a fraction of PKC-δ and -θ attached to the membrane and concentrated in the Golgi apparatus. However, within minutes of adding phorbol ester, there occurs an extensive redistribution. The -α and -ε isoforms concentrate at the cell margin, -a in the endoplasmic reticulum, β_2 associates with actin-rich microfilaments of the cytoskeleton, -γ in Golgi, and -ε attaches to the nuclear membrane (Goodnight et al., 1995). This differential redistribution effectively dictates access to isoform-specific substrates and ultimately confers functional selectivity and so counteracts the basic lack of substrate selectivity among the various protein kinase isotypes. The mechanisms that direct the localization are not fully understood.

DIFFERENT TYPES OF PROTEIN KINASE C-BINDING PROTEINS

Subcellular localization is determined by an array of protein kinase C-binding proteins. Their nomenclature is muddlesome and they have been called anchors, scaffolds, and receptors. Initially, distinction was made between those that interact with active (RACK) or inactive PKC (PICK) and those that are or are not substrates (STICK). The primary function of all these “binding” proteins is to position individual protein kinases into the precise locations in which they can respond to specific receptor-mediated activating signals and address their substrates. Given the promiscuity of protein kinase C in test tube kinase assays, these proteins may act to prevent inappropriate phosphorylation events in the cellular environment. A similar theme emerges with the serine/threonine protein phosphatases (see Chapter 18, “Protein Phosphatases”).

RACKs

RACK1, receptor for activated C-kinase, is characterized by seven WD repeats, having a β-propeller architecture similar to the β-subunits of G protein (return to Chapter 2, “An Introduction to Signal Transduction,” Figure 2-30)

(Ron et al., 1994). Of these WD2, three and six interact with the C2 domain or with the C-terminal region of protein kinase C (predominantly -α, -β, -δ, and -ε). By binding the C2 domain, the RACKs could act to maintain the open active conformation of protein kinase C (Kheifets and Mochly-Rosen, 2007). They are thought to anchor activated PKC to particular membrane domains in the vicinity of appropriate substrate proteins. RACK1 links protein kinase to cytoplasmic tails of a large range of receptors and ion channels, for instance it connects PKCβ with the intracellular domain of the receptors for interleukin-5 and with the γ2-subunits of GABA_A (Geijsen et al., 1999; Feng et al., 2001; Adams et al., 2011). However, RACK1 is also a component of the 40S ribosomal subunit, localized near the head region where mRNA exits the ribosomal particle. RACK1-mediated recruitment of active protein kinase C stimulates translation through phosphorylation of the initiation factor eIF-6 and possibly of mRNA-associated proteins (Figure 9-9) (Nilsson et al., 2004). We ignore entirely how protein kinase C remains in an active state at this localization, in particular in view of the finding that RACK1 binds the (normally) membrane-bound C2 domain? Since RACK1 also interacts with membrane-bound receptors, including integrin β-subunits, it promotes the docking of ribosomes at sites where local translation is required, such as focal adhesions and the tips of extending neural growth cones.

Protein kinase C does not have the exclusive right to bind RACK1, the protein is rather promiscuous. It is a binding partner of the tyrosine protein kinase SRC as well as the serine/threonine protein kinases BRAF, MAP2K1 (MEK1), MAP2K7, MAPK3 (ERK1), MAPK10 (JNK), MAPK11 (p38), and the cAMP phosphodiesterase PDE4D (Li and Xie, 2014). Moreover, in a yeast proteomic study it was shown that RACK1 (Asc1p in yeast) controls the biosynthesis of transcription regulators that modulate signaling pathways (such as the MAP-kinase pathway) (Rachfall et al., 2013). In short, RACK1 is everywhere.

Another RACK is COPB1 (β-COP), a protein with HEAT domains, which interacts with active PKCε in cardiomyocytes, linking it to the Golgi membrane (Csukai et al., 1997). Coatomer proteins, or COPs, play a role in vesicle formation and retrograde transport between *cis*-Golgi cisternae and the ERGIC compartment. They are recruited to the Golgi-membrane through the monomeric GTPase ARF1.

STICKs, substrates that interact with inactive protein kinase C

Examples of substrates that interact with protein kinase C in its inactive state are vinculin, talin, MARCKS, α- and γ-adducin, and the annexins. All these play roles in linking the actin cytoskeleton with the plasma membrane. This implies that protein kinase C is a regulator of membrane–cytoskeletal interactions, in particular at adherens and tight junctions (Larsson, 2006). None of these is selective between different protein kinase C isoforms.

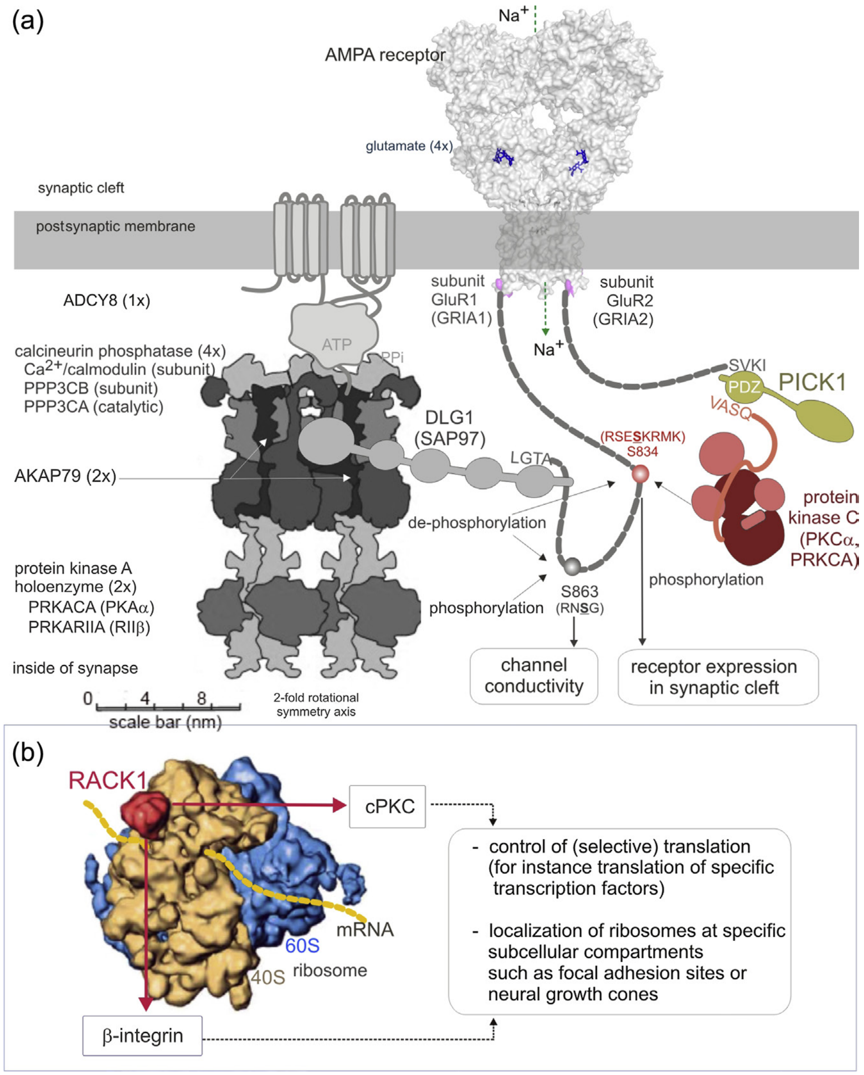


FIGURE 9-9 PICK1 and RACK1-mediated targeting of protein kinase C. (a) PICK1 binds to the extreme C-terminal (SVKI) of the AMPA receptor subunit GluR2 and recruits protein kinase C to the glutamate-gated ion channel. In doing so it brings the protein kinase in the vicinity of serine-834 (in the sequence RSESKRMK). Phosphorylation of this residue facilitates retention of the AMPA receptor in the synaptic cleft and as a result reinforces synaptic current. We deliberately have included the protein kinase A signaling cassette, recruited by DLG1, to illustrate the complexity of signaling events. Phosphorylation of S863 (in the sequence RNSG) augments ion conductivity of the channel. For more information about the role of protein kinase A, return to Chapter 7, Figure 7-7. (b) RACK1 recruits conventional protein kinase C to the ribosome and acts to coordinate events arising from conventional PKCs that may have an effect on the translation rate. The eukaryotic initiation factor-6 (eIF6) is a substrate. RACK1 also binds β-integrins enabling it to localize ribosomes to subcellular compartments. *Image adapted from Nilsson et al. (2004).*

PICKs, proteins that interact with inactive protein kinase C

Anyone who has tried unsuccessfully to swat a fly must realize that the speed of their visual response is faster by far than ours. More than this, the sensitivity of their photosensors and the capacity to adapt, far outstrips that of any vertebrate. The phototransduction cascade in *Drosophila* reveals how scaffold proteins operate in signal transduction through protein kinase C (return to Chapter 5, "Sensory Signal Processing; Visual Transduction and Olfaction," Figure 5-13). Most of the proteins that operate in the phototransduction cascade associate as a single supramolecular signaling complex through PDZ-mediated protein–protein interactions and this contributes to the speed of reaction. The key component in this complex is InaD having multiple PDZ domains, of which PDZ-2 binds to protein kinase C (known as InaC in *Drosophila*). InaD qualifies as a protein binding to inactive C-kinase (PICK). Another protein, human PICK1, also employs its PDZ-domain to bind to protein kinase C. In the example of the AMPA receptor (a heteromeric complex of glutamate receptors, for instance GluR1 and GluR2), STICK1 binds the extreme C-terminal sequence (SVKI) of the GluR2 subunit. It then recruits protein kinase Cα through binding to its extreme C-terminal sequence QSAV. With the appropriate stimuli, this leads to phosphorylation of ser-834 (within the sequence RSESKRMK) of the GluR1 subunit (Staudinger et al., 1997; Collingridge et al., 2004). As a result more AMPA receptors remain in the synaptic cleft and this contributes to an enhanced synaptic signal transmission (involved in the process of long-term potentiation) (Figure 9-9) (Boehm et al., 2006). Return to Chapter 7, "Bringing the Signal into the Nucleus; Regulation of Gene Expression" (Figure 7-7 and to Chapter 8, "Nuclear Receptors" (Figure 8-18) for more information about synapses, AMPA receptors and long-term potentiation in the context of learning).

Proteins that interact with atypical protein kinase C

As already mentioned, atypical protein kinase C isoforms contain a PB1 domain that interacts with other PB1-containing proteins such as SQSTM1 (ZIP/p62), MAP3K2 (MEKK2), MAP2K5 (MEK5), or PARD6 (PAR-6) (Moscat et al., 2006) (return to Figure 9-5). Of these, SQXTM1 recruits atypical protein kinase C into TNF-α and IL-1 receptor complexes and contributes to the activation of the NFκB pathway through the phosphorylation and activation of the protein kinase IKK. Moreover, PKCζ phosphorylates RelA (a component of NFκB complex) in the NLS motif so promoting its transcriptional activity (Moscat et al., 2007). SXQTMA also links PKCζ with the potassium channel subunit KCNAB2 (Kvβ2) and to the growth factor receptor-bound protein 14, Grb14. PAR-6, to which we return later, links atypical PKC to CDC42, a monomeric GTPase of the Rho family of GTPases, involved in the regulation of cell polarity.

HOLDING BACK THE PKC RESPONSE

Diacylglycerol kinases

Conversion of diacylglycerol to phosphatidic acid provides the means to deactivate conventional and novel protein kinase C. The family of diacylglycerol kinases (9 members) is subdivided on the basis of either substrate specificity or the presence of specific domains. They all have C1 or extended C1 domains (15 extra amino acids) and they all have catalytic and accessory domains (see also PI 3-kinase: Chapter 16). Despite the presence of a C1 domain, only diacylglycerol kinases β and γ interact with phorbol ester. The type I diacylglycerol kinases (comprising α, β, and γ) are characterized by two Ca^{2+}-binding EF hands. Type II kinases (comprising δ and η) are characterized by PH and SAM domains. The SAM allows binding to the endoplasmic reticulum and plays a role in vesicle trafficking. Type III (only ε) is without recognizable additional domains. Type IV kinases (comprising ζ and ι) have a MARCKS-homology sequence which acts as a NLS. Type V (only θ) possesses three C1 domains.

Although conclusive evidence is still lacking, the diacylglycerol kinases are believed to be quiescent until their activity is required. This is achieved by recruitment to the membrane through binding cofactors and phosphorylation, by for instance, activated protein kinase C (van Baal et al., 2005).

Phosphatidic acid

Although diacylglycerol kinase removes diacylglycerol and thus terminates membrane localization of protein kinase C, it does not necessarily stop membrane signaling because the product phosphatidic acid itself has numerous effects. It stimulates DNA synthesis, helps recruitment of BRAF to the membrane, and modifies the activity of RasGAP and of PKCζ (which does not respond to diacylglycerol) (return to Figure 9-7) (Topman, 2006).

Down-regulation by phorbol esters

Prolonged activation by phorbol esters results in a loss of protein kinase C activity. This is the consequence of both dephosphorylation of critical residues and degradation of the protein. Prior treatment of cells with phorbol ester, leading to depletion of a number of enzyme isoforms, has been extensively applied in studies aimed to test a role for protein kinase C responses to physiological stimuli.

PROTEIN KINASE C IN THE CONTEXT OF ONCOGENIC TRANSFORMATION

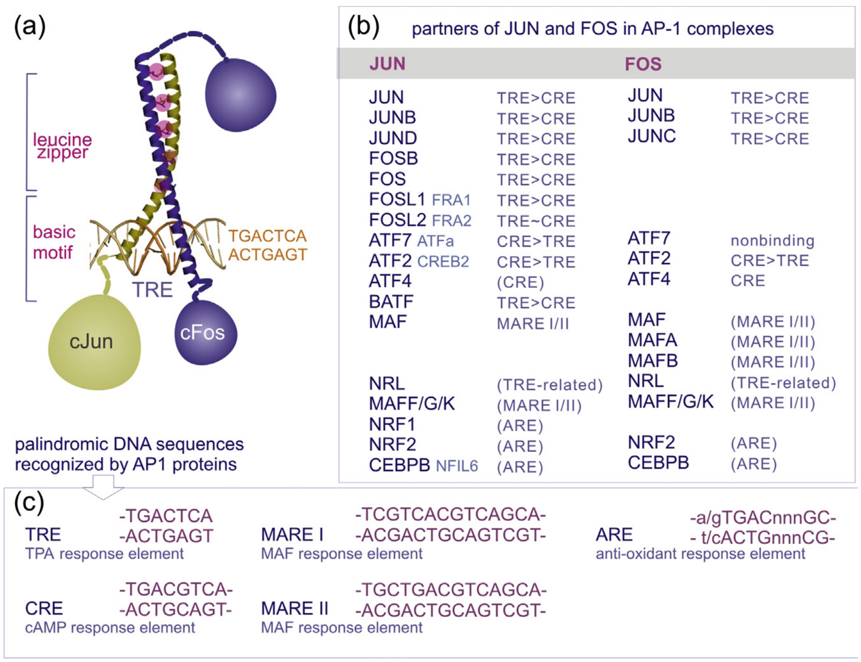
Long before their association with protein kinase C became apparent, it was evident that extracts of croton oil can act as co-carcinogens, enhancing the tumorigenic activity of other substances such as benzo(a) pyrene (Berenblum, 1941). In some culture systems phorbol ester increases the number of mitoses (Sivak and van Duuren, 1967) but in the absence of a tumor promoter, it fails to effect full cell transformation. One might think that protein kinase C should be implicated in all this, maybe acting to phosphorylate a transcription factor to induce specific gene expression.

Benzopyrene is a highly carcinogenic five-ring polycyclic aromatic hydrocarbon. It is the component of coal tar responsible for the cancers (sooty warts, cancers of the scrotum) suffered by chimney sweeps in eighteenth century and fuel industry workers in nineteenth century England and to this day it presents a health hazard as a carcinogen present in cigarette and marijuana smoke and barbecued hot dogs (Cook et al., 1933). As with many other chemical carcinogens benzopyrene is better regarded as a procarcinogen, requiring conversion to an active derivative, benzo[a]pyrene diol epoxide. This slots into the major groove of DNA and basepairs with thymine at the replication junction and this may facilitate the generation of mutants in the process of lesion bypass DNA synthesis (causing frame-shift mutations or base substitution) (Ling et al., 2004).

With the aim of understanding the mechanisms underlying tumor promotion by phorbol esters, two independent strategies have been applied. By searching for transcriptional control elements that mediate the phorbol ester-induced alterations in gene expression, it should be possible to work backwards, identifying first the transcription factor(s) that bind these elements and then the signal transduction pathway that regulates their activation (Imbra and Karin, 1986). The alternative approach has been to overexpress various isoforms of PKC and to study changes in cell phenotype (Housey et al., 1998). Despite a truly voluminous bibliography, understanding of the relationship between protein kinase C and tumor formation remains somewhat hazy. As we will learn below, protein kinase C plays accessory roles, modulating the activity of pathways controlled by established transformers such as mutated growth factor receptors, RAS and RAF.

The discovery of TRE, AP-1 and how mitogen-activated protein kinases stole the limelight

The search for transcription factors started with the analysis of promoter regions of a number of genes (for instance, collagenase, metallothionein IIA, and stromelysin) induced by phorbol ester. It revealed a conserved seven base pair palindromic motif, named the TPA-response element (TRE) (Figure 9-11). The DNA sequence is recognized by activator protein-1 (AP-1), understood to be at the receiving end of a complex pathway that transmits the effects of phorbol ester tumor promoters from the plasma membrane to the transcriptional machinery, downstream of protein kinase C (Lee et al., 1987; Angel et al., 1987). Today, AP-1 no longer refers to a single TRE-binding protein but to a group of structurally and functionally related members of the JUN and FOS protein family together with some members of the ATF and MAF families (in combination with Jun proteins). They are part of the bZIP family of transcription factors recognized by a basic motif for DNA binding and a leucine zipper that engenders dimerization (Figure 9-10) (Sassone-Corsi et al., 1988). For more information about the bZIP family,



(b) partners of JUN and FOS in AP-1 complexes

JUN		FOS	
JUN	TRE>CRE	JUN	TRE>CRE
JUNB	TRE>CRE	JUNB	TRE>CRE
JUND	TRE>CRE	JUNC	TRE>CRE
FOSB	TRE>CRE		
FOS	TRE>CRE		
FOSL1 FRA1	TRE>CRE		
FOSL2 FRA2	TRE~CRE		
ATF7 ATFa	CRE>TRE	ATF7	nonbinding
ATF2 CREB2	CRE>TRE	ATF2	CRE>TRE
ATF4	(CRE)	ATF4	CRE
BATF	TRE>CRE		
MAF	MARE I/II	MAF	(MARE I/II)
		MAFA	(MARE I/II)
		MAFB	(MARE I/II)
NRL	(TRE-related)	NRL	(TRE-related)
MAFF/G/K	(MARE I/II)	MAFF/G/K	(MARE I/II)
NRF1	(ARE)		
NRF2	(ARE)	NRF2	(ARE)
CEBPB NFIL6	(ARE)	CEBPB	(ARE)

(c)

TRE -TGACTCA -ACTGAGT
TPA response element

MARE I -TCGTCACGTCAGCA- -ACGACTGCAGTCGT-
MAF response element

ARE -a/gTGACnnnGC- - t/cACTGnnnCG-
anti-oxidant response element

CRE -TGACGTCA- -ACTGCAGT-
cAMP response element

MARE II -TGCTGACGTCAGCA- -ACGACTGCAGTCGT-
MAF response element

FIGURE 9-10 Activator protein-1 (AP-1) complexes and their cognate response elements. (a) Structure of the leucine zipper and basic motif of JUN and FOS, both members of AP-1 complex, bound to the palindromic sequence TGACTCA. (b) Partners of JUN and FOS in AP-1 complexes (JUN/JUN, JUN/FOSL1, and FOS/JUN, FOS/ATP7, etc). (c) Examples of palindromic DNA sequences recognized by AP-1 transcription factors.

return to Chapter 7, “Bringing the Signal into the Nucleus,” Figure 7-9. The AP-1 transcription factors are similar in structure to CREB (Karin et al., 1997; Bohmann and Tjian, 1989). Other response elements recognized by AP-1 are CRE, MARE I, MARE II, and ARE. A list of possible combinations with their preferred DNA response element is presented in Figure 9-10. Although originally discovered in the context of tumorigenesis, not all forms of AP-1 cause cell transformation and their effect is dependent on context. Roughly speaking, FOS, FOSB, and JUN (c-jun) are considered oncogenes although they have not been found associated with cancer (no mutants). FOLS1 (Fra1) and FOSL2 (Fra2) are considered weak oncogenes (see below) whereas JUNB and JUND have tumor suppressor qualities (Eferl and Wagner, 2003).

AP-1 The activator protein-1 transcription factor complexes owed their initial definition to a fortuitous convergence of tumor virology and transcription factor research. AP-1 was discovered as a novel activator protein that drives expression of the human metallothionein genes. It also binds 72-base pair repeats of the simian virus 40 (SV40) enhancer region (Lee et al., 1987). Both the methallothionein and the SV40 enhancer region contain a similar conserved sequence motif (of seven base pairs) that was also found in other phorbol-ester inducible genes and was named TRE. Indeed, the DNA sequence binds a 44 kDa protein, identical to the protein characterized as the transcription factor AP-1 and later identified as c-Jun (JUN) (Bohmann et al., 1987).

The oncoproteins v-Fos and v-Jun were identified as transforming proteins in the Finkel–Biskis–Jinkins murine osteosarcoma virus and in avian sarcoma virus-17, respectively. v-Jun was linked with AP-1 because of its homology with the yeast transcriptional regulator GCN4, which binds an identical DNA sequence. Indeed, the DNA-binding domains of Jun and of GCN4 are exchangeable. The cellular homolog of v-Fos associates with a protein of 39 kDa, recognized as c-Jun. This finding identified FOS and JUN as partners, together forming the AP-1 complex that binds DNA (Vogt, 2001). The viral proteins, v-Fos and v-Jun, are transforming proteins by virtue of their prolonged half-lives, enhanced transcriptional activity, and a change in the spectrum of target genes (compared to their cellular counterparts) (Bohmann and Tjian, 1989).

The upstream signal transduction pathway that regulates AP-1 activity proved a hard nut to crack. Initially it appeared that activation of protein kinase C causes dephosphorylation of c-Jun, just in the basic motif where it binds DNA (Boyle et al., 1991). Phosphorylation of this segment can also be achieved (in the test tube) by glycogen synthase kinase-3β (GSK) and so it was postulated that protein kinase C stimulates the binding of c-Jun DNA through inhibition of GSK-3β which would result in dephosphorylation of the basic region. Consistent with this is that activation of protein kinase C (α, β1, β2, and γ) causes phosphorylation and thus deactivation of GSK-3β (Goode et al., 1992). However the interaction between GSK-3β and JUN (c-Jun) needed verification and the phosphatase that strips the phosphates remained enigmatic. That this could not be the whole story became clear when it was found that phosphorylation of four residues at the N-terminus is far more important for c-Jun-mediated transcriptional activity and cell transformation (Smeal et al., 2000; Behrens et al., 2000). The discovery of a Jun N-terminal protein kinase, JNK1 or MAPK8, drew attention away from protein kinase C and onto a much more productive research field dealing with the newly emerging family of mitogen (growth factors in serum)-activated protein kinases (MAP kinases) (Derijard et al., 1994; Kallunki et al., 1996). Many of the mitogen-activated protein kinases exert their nuclear response through yet another DNA-response element, named the serum response element (Treisman, 1985, 1986). When, around 1993, it was shown that the proto-oncogene RAS acts downstream of growth factor receptors and upstream of mitogen-activated kinases, the interest for mitogen-activated kinase largely prevailed (Figure 9-11) (Buday and Downward, 1993).

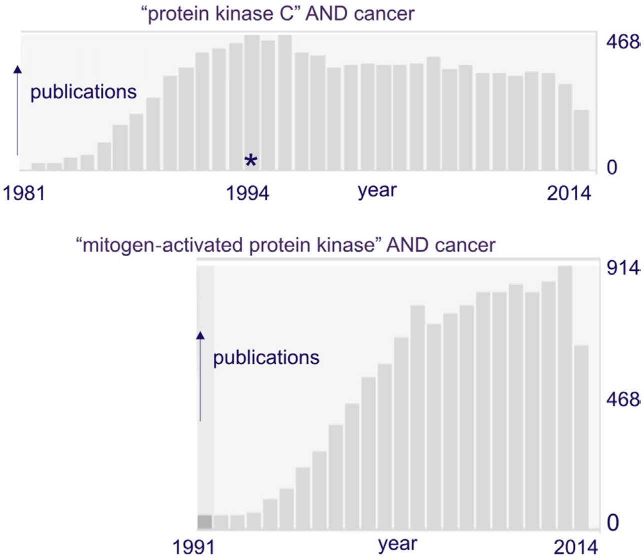


FIGURE 9-11 Shifting interest from protein kinase C to mitogen-activated protein kinases after the discovery of RAS-mediated signaling events downstream of growth factor receptors around 1993 (*).

Overexpression of PKC isoforms and cellular transformation

The second approach in the quest of understanding the tumor-promoting action of phorbol esters consisted of enhancing protein kinase C-activity through ectopic expression. Excessive expression of PKC-β1 (PRKCB) in rat embryo (R6) fibroblasts induces the characteristic transformed cell phenotype. What this means is that the cells have a reduced requirement for growth factors and generate an autocrine mitogenic factor that possibly induces their own transformation. Other parameters of transformation are the lack of contact inhibition and the capacity to grow under conditions with limited adhesion contacts (in soft agar). However, when injected into nude mice, these cells induce tumors having a lower frequency and longer latency period than cells transformed by the Ha-RAS oncogene (Borner et al., 1995). This should not be entirely unexpected with phorbol esters acting as tumor promoters or co-carcinogens, not carcinogens in their own right. Coexpression of Ha-RAS and PKC-β1 certainly results in greatly enhanced transformation (Hsiao et al., 1989). In contrast, for R6 cells overexpressing PKC-α (PRKCA) there is no tendency to transform (Borner et al., 1995). Instead, growth is retarded and the cells achieve lower saturation densities than normal. In glioma cells, PKC-α has a growth-promoting role, suppressing the expression of the cell-cycle inhibitor $p21^{WAF1/CIP1}$ (Besson and Yong, 2000). A summary of phenotypic changes caused by ectopic expression of members of the PKC family is presented in Table 9-1.

(Atypical) PKC-ζ (PRKCZ) has tumor-suppressor qualities actually reverting v-Raf transformation of NIH-3T3 cells (Kieser et al., 1996) and (novel) PKC-δ (PRKCD) inhibits proliferation of vascular smooth muscle cells by suppressing the expression of the cyclins D and E, components of the cell-cycle machinery that prepare the cell for DNA synthesis (Fukomoto et al., 1997). It appears that depending on the cells and on the circumstances, different isoforms of PKC, when overexpressed, can either induce or suppress the formation of the transformed cell phenotype. Strong evidence against a crucial role of protein kinase C in human tumorigenesis comes from the finding that few natural tumors have been found that express protein kinase C mutations. There is but a lone example, a point mutation in PKCα in a subpopulation of highly invasive pituitary tumors and in thyroid follicular adenomas and carcinomas (Prevostel et al., 1997, 1998). Importantly, as already indicated, there are many other proteins that possess C1-domains similar to those present in the protein kinase C that may also be regulated by phorbol esters. One of these, CalDAG–GEF, is a guanine nucleotide exchange factor for Ras (and Rab), which promotes malignant transformation in fibroblasts when induced by phorbol ester (Tognon et al., 1998). Besides a possible role in cancer, protein kinase C has been implicated in pathologies related to diabetes and it has been reported to play a role in heart failure, myocardial infarction, pain, and bipolar disorder.

An extensive review dealing with protein kinase C, its implication in disease, and how specific inhibitors have yet failed to reach therapeutic potential (Mochly-Rosen et al., 2012).

TABLE 9-1 Phenotypic changes caused by ectopic expression of different isoforms of PKC

PKCα	PKCα	PKCβ1	PKCδ	PKCε	PKCζ
Rat colonic epithelial cells	Glioma cells	Rat embryo fibroblasts (R6)	Vascular smooth muscle cells	Rat fibroblasts and colonic epithelial cells	v-raf transformed NIH-3T3
Tumor suppression	Tumor promotion	Tumor promotion	Tumor suppression	Tumor promotion	Tumor suppression
• Slower growth • Low densities	• Suppression of p21$^{WAF/CIP}$	• Growth in nude mice • Anchorage independence • Synergy with Ha-RAS	• Slower growth • Reduced expression of cyclin D and E	• Growth in nude mice • Anchorage independence • Synergy with RAS	• Reversion of transformed phenotype

Protein kinase C contributes to cell transformation by driving the epithelial–mesenchymal transition

Cancer stem cells

Although it has not been possible to draw any strong generalizations about the role of protein kinase C in cell transformation, a number of protein kinase C-mediated events has been described that show a clear oncogenic potential and we will illustrate some of these below. It is generally considered that cancer arises from cancer stem cells. It is important to know that tissue stem cells are functionally defined, they are not recognized by a specific transcriptome (molecular signature) as is the case for embryonic stem cells (Sato et al., 2003). Thus tissue stem cells are defined as cells that are able to regenerate at clonal level (one cell only) a full tissue by producing its differentiated cellular components. The definition holds true for cancer stem cells in that they are defined as being able to regenerate the initial cancer. In general stem cells occupy 1–3% of the cell population concerned. The origin of cancer stem cells is not clear. It is likely that they arise from tissue stem cells or early progenitor cells for the simple reason that their "lifelong" proliferation offers the best opportunity to accumulate sufficient mutations that ultimately cause cell oncogenic transformation. The mutation rate of cells is estimated to be roughly three per division in a "stable" genome and around 300 in an "instable" genome. Instable genomes are the consequence of a loss of function of proteins that either control the integrity of DNA or control the fidelity of replication, among them, ATM, BRCA1, MSH2, and TP53 (Stratton et al., 2009; Dujon, 2005). Nonsilent gene mutations often lead to cell death or cause senescence, but depending on the type and the order of mutations, one or two stem cells may survive and transmit the phenotypic consequences of their defective genome to the progenitors (process of clonal evolution). When these cells have a proliferative advantage they will gradually take over the tissue. Although gene mutations are naturally all shared between stem cells and their progeny, this is not necessarily the case for phenotypic traits. Transformed stem cells, also known as cancer stem cells, may conserve the capacity to divide asymmetrically and maintain their exclusive stem cell status by withholding from their progeny essential proteins and RNAs. Return to Chapter 7, “Bringing the Signal into the Nucleus,” Figure 7-1 for an overview of the different types of RNA. Later in the chapter we will return to asymmetric division. The epigenetic program of the progenitor cells therefore will be different from the stem cells and indeed it has been reported that progenitors are not necessarily as tumorigenic as their stem-cell parents (Schackleton et al., 2009). As a consequence of this and because of varying cell–cell interactions between transformed progenitors and the tissue environment, just as in healthy tissues, cancer cell populations are a mixture of cells with different traits. However, unlike cells in healthy tissues, this collection of cells proliferates excessively, takes on inappropriate functions, and has the capacity to breach anatomical barriers (metastasize). Indeed, epithelial cells obtained from breast cancer tissue fail to enter a full differentiation program and resemble more poorly differentiated cells of the basal lineage with mesenchymal characteristics (Sarrio et al., 2008). The phenomenon of epithelial cells reverting to a mesenchymal state is named epithelial–mesenchymal transition (EMT). It is observed at various stages in embryonic development where the transition, which is normally transient, is characterized by a loss of cell–cell contact, a switch from epithelial to mesenchymal markers (see below), and a capacity to migrate. This process occurs, for instance, during gastrulation, where cells from the epiblast (ectoderm) detach and migrate inward to establish a mesodermal layer (Nakaya and Sheng, 2013; Martin-Belmonte and Perez-Moreno, 2012).

The existence of cancer stem cells has first been recognized in myeloid leukemia (Bonnet and Dick, 1997). Now we know that they occur in breast, colon, head and neck, lung and prostate carcinomas (malignant epithelial cancers) (Ailles and Weissman, 2007). We note that tumors may not necessarily be the result of the clonal evolution of a single stem cell. The presence of multiple cancer stem cells is not necessarily excluded and these may occur at different anatomical locations, each with their own history of mutations and their particular cellular environment. (Rey et al., 2001). This gives rise to even further cancer-cell heterogeneity (hence complicate therapeutic intervention) (Visvader, 2011).

STEM CELLS AND INDUCED STEM CELLS

Whether organisms are maintained by a selected set of stem cells that accompany them for a lifetime or are maintained by a mixture of "genuine" stem cells and differentiated (mature) cells that have reverted to stem-cell status is currently not known. The finding that differentiated cells can revert to pluripotent stem cells (so-called "induced stem cells") and even give rise to a whole new organism (procedure known as cloning, made famous with the birth of "Dolly") has turned upside down our understanding of genomic plasticity. The Nobel Prize in Physiology or Medicine 2012 was awarded to John Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent" (Gurdon, 1962; Campbell et al., 1996; Takahashi and Yamanaka, 2006).

As cancer stem cells may be the forerunners in genomic errors they are also likely to be the forerunners in acquiring resistance; they may have accumulated specific mutations that offer them the privilege of being able to pump medicaments out of the cell, to modify the therapeutic targets so that they are no longer recognized or to mobilize metabolic pathways that inactivate the medicaments. Importantly, stem cells are known to express pumps, for instance ABCG2, and these may play a role in the exclusion of medicaments (Ding et al., 2010). As a consequence, cancer stem cells may be more likely to escape chemotherapy. Moreover, despite them being transformed, cancer stem cells may still divide less frequently than their progeny. It has been shown that committed progenitors in healthy tissues tend to undergo a transient burst of division cycles before settling down as differentiated cells. Again, this may facilitate survival of cancer stem cells because cancer treatment is limited to a very narrow therapeutic window (in the order of 1 or 2 days repeated over a 6-month period). These survivors then cause cancer recurrence, but this time clonally selected for resistance.

Cancer stem cells and protein kinase C

Cancer stem cells, like healthy stem cells, constitute a minute population within the tissue and this makes them hard to study. It would be helpful if they could be reconstituted from a large cell population. We show the example of how immortalized (but not transformed!) mammary epithelial cells can be converted into cells with properties of stem cells and from there into cancer stem cells (Mani et al., 2008). Protein kinase C takes central stage in all this. The first stage toward oncogenic epithelial stem cells comprises the induction of EMT by ectopic expression of the transcription factor Snail (SNAI) or Twist (TWIST). We return to these genes in Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation," Table 12-1 and Figure 12-15. They cause a gain of expression of cellular mesenchymal markers such as vimentin (VIM, intermediate filament), N-cadherin (CDH2) and cadherin-11 (CDH11), an enhanced production of extracellular matrix components SPARC, laminin (LAM-A, -B, and -C), fibronectin (FN1), and fascin (FASN1–3). They also cause the loss of epithelial markers such as E-cadherin (CDH1) and cytokeratins (KRT-8, -18, and −19, intermediate filaments). In a naturally occurring EMT, expression of the transcription factors SNAI1 (Snail), SNAI2 (Slug), and TWIST1 is also elevated (Sarrio et al., 2008).

The transition from an epithelial to mesenchymal state is accompanied by an elevated expression of the receptor for platelet-derived growth factor (PDGFRA, -B), protein kinase PKCα (PRKCA), and phospholipase C-γ (PLCG1, -2) as well as an up-regulation of expression of the AP-1 member of transcription factors Fra1 (FOSL) but a down-regulation of c-Fos (FOS). Moreover, the treated cells where predominantly CD44high and CD24low, both of which are markers of basal stem cells in mammary glands (for the anatomy of the mammary gland, return to Chapter 8, "Nuclear Receptors," Figures 8-16 and 8-17). Compared to the parental epithelial cells, the SNAI1- or TWIST-treated cells formed mammospheres (semi-organized glandular structures in cell culture) more efficiently than parental cells, which is indicative of a stem cell–enriched cell population. The cells that have undergone an EMT do not yet fully qualify as cancer stem cells, but they are much more prone to become so with a growth-promoting mutation such as that occurs in RAS (oncogene). Importantly, and here comes a key role for protein kinase C, the treated cells were highly susceptible to inhibitors of PKCα, nearly 90% died in their presence, compared to only 22% for the parent epithelial cells (Tam et al., 2013). The scheme that emerges is that activation of phospholipase C by PDGF, acting in an autocrine fashion, leads to the production of diacylglycerol and an increase in intracellular free Ca^{2+}, thus activating PKCα (Figure 9-12). In parallel, expression of SNAI or TWIST leads to enhanced expression of FOSL which teams up with JUN in an AP-1 complex that binds to enhancer elements that further drive the stem-cell program. Indeed, high levels of JUN/FOSL have been detected in highly metastatic basal-like mammary gland cell lines (Baan et al., 2010). Inhibition of PKCα or PDGFR thus constitute novel targets that could help to more effectively eliminate cancer stem cells and thus serve as a complement to current therapeutic approaches in breast cancer. Below we will show in more detail how protein kinase C activates components of the AP-1 complex and contributes the maintenance of a mesenchymal state.

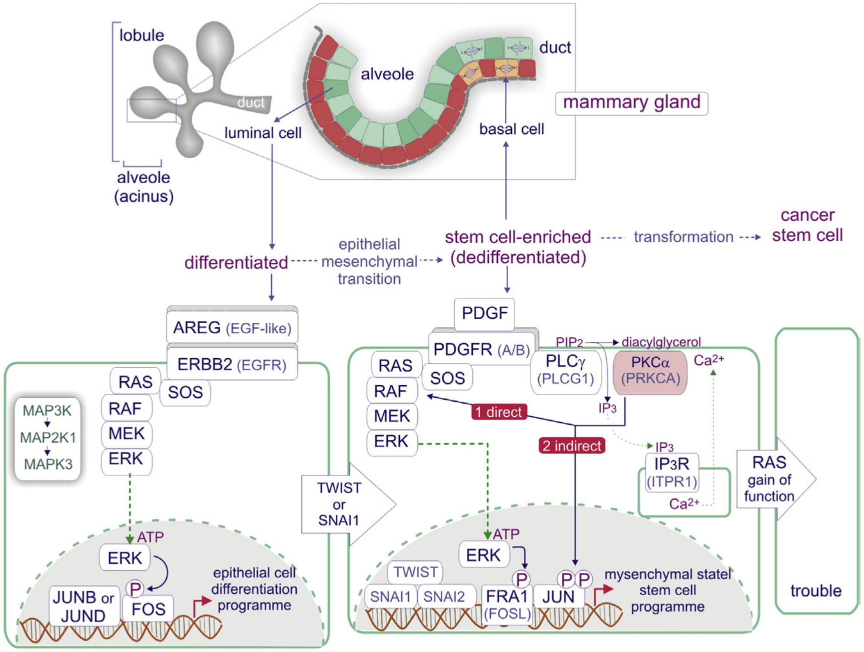


FIGURE 9-12 Role of protein kinase C in driving the cancer stem-cell program. Mammary gland stem cells are found in the basal cell layer in ducts. These stem cells give rise to basal (myoepithelial) and luminal (milk producing) committed progenitor cells. Cells of the luminal lineage are epithelial cells and more differentiated in nature. They are regulated by the AP-1 (activator protein-1) transcription factors, FOS, and JUNB/JUND. Ectopic expression of TWIST or SNAI1 (Snail) causes a switch in the phenotypic cell state that is known as the epithelial–mesenchymal transition (EMT). This generates cells bearing a mesenchymal phenotype that represents a de-differentiated state and they bear resemblance of basal stem cells. These cells are characterized by an autocrine platelet-derived growth factor (PDGF) signaling, which leads to the expression and activation of an alternative AP-1 complex, comprising FRA1 (FOSL) and JUN (c-JUN). They govern a stem-cell transcription program. Protein kinase C plays a crucial role by boosting the RAF pathway, leading to phosphorylation and activation of FRA1, and by boosting phosphorylation and activation of JUN. Inhibition of PKCα causes cell death, hinting to an essential role in maintaining the stem-cell program. Oncogenic stem-cell transformation requires another "hit," for instance, a gain of function mutation in the monomeric GTPase RAS (oncogene). This leads to the generation of cancer stem cells (CSC), recognized by their capacity to reconstitute similar tumors in xenografts or in ex vivo conditions. Mammary gland tumors, also known as breast cancer, often originate from duct basal cells (return to Chapter 8, "Nuclear Receptors," section "Regulation of Mammary Gland Stem-Cell Proliferation Through Paracrine Signaling"). The abbreviations in green are added to illustrate the standard composition of a mitogen-activated kinase cassette in which a MAP3K phosphorylates and activates a MAP2K which in turn phosphorylates and activates a MAPK. Note that the composition of these cassettes varies, see also Figure 9-14. *Image adapted from Tam et al. (2013).*

Protein kinase C activates the mitogen-activated kinase pathway through RAF

Both expression and activity of FOSL are normally controlled by mitogen-activated protein kinases and not by protein kinase C (Vial et al., 2003; Kakumoto et al., 2006). Yet studies with selective protein kinase-C inhibitors show that it plays an essential role because treatment causes a rapid loss of phosphorylation of FOSL on ser-265. The question arises of how protein kinase C controls activity of the mitogen-activated pathway? The mitogen-activated pathway we refer to involves the monomeric GTPase RAS, which controls a cassette of protein kinases in the order of RAF–MEK–ERK (in gene names: RAF1–MAP2K1–MAPK3) (Figure 9-13). We return extensively to these protein kinases in Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases." Protein kinase C has no effect on RAS but is capable of stimulating the mitogen-activated protein kinase pathway (Schonwasser et al., 1998) through the activation of RAF in two fashions. Firstly, conventional protein kinase C (α,β, and γ) directly phosphorylates RAF1 (or BRAF) in a series of residues in the activation segment (sequence

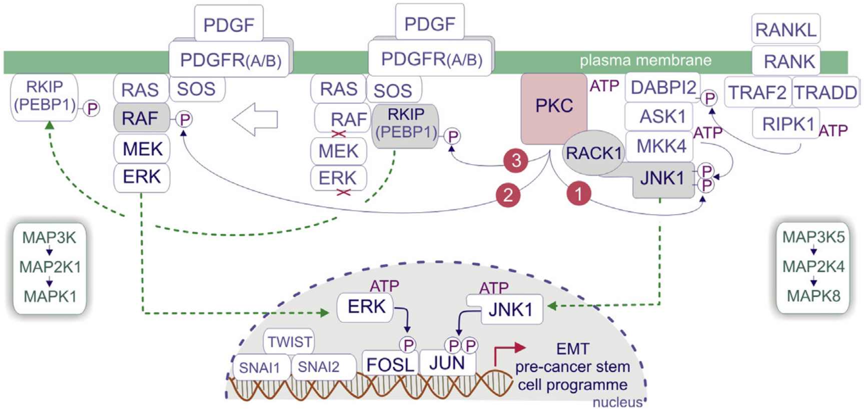


FIGURE 9-13 Protein kinase C and its role in boosting growth-factor signaling. Protein kinase C (without precision of isoforms) can boost mitogen-activated pathways in different fashions. Firstly, through binding of the receptor of activated C-kinase (RACK1) it connects to Jun N-terminal kinase (JNK1 or MAPK8) and assists in phosphorylation of the activation segment. Additional phosphorylation through MKK4 is required. MKK4 normally acts downstream of inflammation/growth factor receptors such as RANK. Once activated, JNK1 will enter the nucleus there to phosphorylate and activate JUN. Secondly, protein kinase C phosphorylates RAF and this too facilitates the activation process which is under control of the monomeric GTPase RAS, by itself controlled by growth factor receptors (PDGFR in the example). Thirdly, protein kinase C phosphorylates the RAF-kinase inhibitory protein RKIP1 (PEBP1) and in doing it detaches from RAF so to facilitate downstream phosphorylation and activation of MEK. The abbreviations in green refer to gene names of the mitogen-activated protein-kinase cassette in question. Note that, unlike all other kinases, RAF keeps it name. It operates at the MAP3K level.

494–SRWSGS–499). Of these, the position of ser-499 was shown to be important for protein kinase C-mediated but not for RAS-mediated activation of RAF1 (in mice) (Sozerie et al., 1992; Kolch et al., 1993). Protein kinase C also boosts RAF signaling by phosphorylating its inhibitor, Raf-kinase inhibitor protein RKIP (PEBP1) (Yeung et al., 1999; Corbit et al., 2003). The inhibitor binds to the phosphorylated N region of Raf-1 and prevents phosphorylation of the downstream protein kinase MEK1. The interaction between the two proteins is abrogated after phosphorylation of RKIP by protein kinase C (Rath et al., 2008). The ensuing activation of the mitogen-activated kinase cassette leads to phosphorylation of FOSL in the nucleus. RKIP has the gene name of PEBP1 which is an abbreviation of phosphatidylethanolamine-binding protein originally discovered in bovine brain. The pocket that binds phosphorylated RAF1 also binds the charged head group of phosphatidylethanolamine or nucleotides (Tavel et al., 2012).

Protein kinase C sensitizes the JNK pathway through RACK1-facilitated phosphorylation of JNK1

The expression and phosphorylation of JUN is also elevated and this involves yet another protein kinase, Jun N-terminal kinase-1 (JNK1, gene name MAPK8). The scheme we describe below was originally discovered in melanoma skin cancers where both elevated levels of JUN and its phosphorylation play a role in the high proliferation rates of the cancer cells (Lopez-Bergami et al., 2007; Kappelmann et al., 2014). The mechanism may apply to other cancers. A large proportion of human melanomas harbors a highly active RAS–MEK–ERK pathway due to gain-of-function mutations in the genes coding for BRAF and/or NRAS. CyclinD (CCND1), which drives progression of the cell division cycle in G1, is also up-regulated. The transcription factors JUN and ATF2, forming an AP-1 complex (return to Figure 9-11), are regulators of cyclinD expression. JUN must be phosphorylated (on ser-63 and -73) in order to be transcriptionally active and this could occur through at least two pathways, the one comprising the RAS–RAF–MEK–ERK, the other comprising MKK4 (or -7) and JNK. Studies with kinase inhibitors showed that ERK and JNK are equal partners in maintaining cyclinD at an elevated level. However, the JNK pathway is not generally activated by mitogenic signaling, responding mainly to stress situations (see Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Figure 10-22) (Gille and Downward, 1999). Again, protein kinase C acts as a modulator of the mitogen-activated protein kinases; it boosts activation of JNK1 and thus promotes the transcriptional activity of JUN. The receptor-activated C-kinase (RACK1), one of the protein kinase-C docking proteins, is important in the process. It binds to activated protein kinase C and together they associate with JNK1. The consequence is phosphorylation of JNK1 (residue not revealed but likely threonine-183). PKCα, -β, and -ε are candidates for this phosphorylation reaction, though other isoforms are not excluded. The phosphorylation enables subsequent phosphorylation (on tyr-185) and activation of JNK1 by the upstream kinase MKK4 to render JNK1 fully competent (Figure 9-13). High levels of JUN-activity in turn increase the expression of RACK1 and the ensuing amplification loop explains, among many other things, how melanoma cells maintain such a high rate of proliferation. Not only this, but elevated levels of RACK1 also protect against apoptosis (Lopez-Bergami et al., 2005) and conversely, depletion of RACK1 sensitizes melanoma cells to UV-induced apoptosis and reduces their tumorigenicity. The above-described series of events is also operational in hepatocellular carcinoma (Guo et al., 2013) and alterations in RACK1 have been reported in different types of human tumors, with high levels of expression in nonsmall-cell lung carcinoma and in colon carcinomas (Berns et al., 2000).

What exactly activates MKK4 in the context of the mammary gland is not determined. We have placed the RANKL receptor (RANK) in this scheme because it is one of the highly expressed genes in mammary gland stem cells (Asselin-Labat et al., 2010) and it is a potent activator of JNK (return to Chapter 8, “Nuclear Receptors,” section “regulation of mammary gland stem-cell proliferation through paracrine signaling”) (Song et al., 1997; Baud and Karin, 2001). The RANK pathway comprises two adaptor proteins TRAF2 and TRADD, a protein kinase RIPK1, a scaffold protein DABPI2, and the mitogen-activated kinase cassette starting with ASK1 (MAP3K5), followed by MKK4 (MAP2K4), and leading to activation of JNK1 (MAPK8) (Figure 9-13) (Nishitoh et al., 1998; Zhang et al., 2004, 2007).

RANKL AND OTHER INFLAMMATORY MEDIATORS

In the situation of breast cancer, levels of RANKL are elevated. RANKL is normally produced by nonstem cells of the epithelium in response to progesterone (return to Chapter 8, “Nuclear Receptors,” see section “regulation of mammary gland stem-cell proliferation through paracrine signaling” and Figure 8-17) (Asselin-Labat et al., 2010). In the process of cancer, an additional production is caused by tumor-infiltrating regulatory T cells (Tan et al., 2011). Although RANKL qualifies as an inflammatory mediator, belonging to the family of tissue necrosis factor (TNF) ligands (Chapter 14, “Chemokines and Traffic of White Blood Cells,” Figure 14-3), it has a role in differentiation (osteoclasts) and proliferation (melanoma cells and mammary gland stem cells). More generally, a cocktail of inflammatory mediators are ever present in the cellular environment. This is certainly the case for cancer cells. They often generate their own autocrine growth factors and

continued

RANKL AND OTHER INFLAMMATORY MEDIATORS *(cont'd)*

these also elicit an inflammatory response with concomitant release of inflammatory mediators by surrounding cells and infiltrating blood-borne cells. This enhances their growth and dissemination because of the increased vascular permeability and general vascular remodeling around inflamed tissue but also because of their role in sustaining the cancer stem cell niche. Just one example, the oncogene Src, a mutated tyrosine protein kinase, was shown to be able to bring about a chronic elevated production of interleukin-6 (IL-6) and its production and release contributes to further cell transformation by a pathway that involves NFκB and the transcription factor STAT3 (Iliopoulos et al., 2009). IL-6 is now considered a key mediator in cancer development (Grivennikov and Karin, 2008). Tumor-associated monocytes and macrophages bring about the same effect. They can interact with cancer stem cells and induce, through juxtacrine signaling via Ephrin/EPhA4, the production of IL-6, IL-8, and GM–CSF. These in turn, in an autocrine fashion, maintain the cancer stem cell state (Lu et al., 2014).

ATYPICAL PROTEIN KINASE C AND THE REGULATION OF CELL POLARITY

Polarity cues

The orientation of the mitotic spindle is essential for the control of cell fate, tissue architecture, and, as a consequence, tissue morphogenesis. Anchoring the astral microtubules to the cell surface and subsequent generation of mechanical forces underlie important, but not unique, mechanisms that orient the mitotic spindle and thus the plane of cell division (Dujardin and Vallee, 2002). Astral microtubules connect to the plasma membrane through so-called cortical landmark deposits. The localization of these landmarks in turn is determined by polarity cues such as the sperm point entry, the presence of growth and differentiation factors (morphogens) or, importantly, adhesion contacts (tight junctions, adherent junctions, and focal adhesion contacts) (Figure 9-14). Factors that orient the mitotic spindle not only determine the position of the daughter cells within the tissue but they also play a role in the asymmetric distribution of protein and RNA. Spindle orientation contributes to cell fate: stem cell versus committed progenitor or epidermal cell versus sensory organ precursor, etc. In this section, we focus on the mechanisms that translate

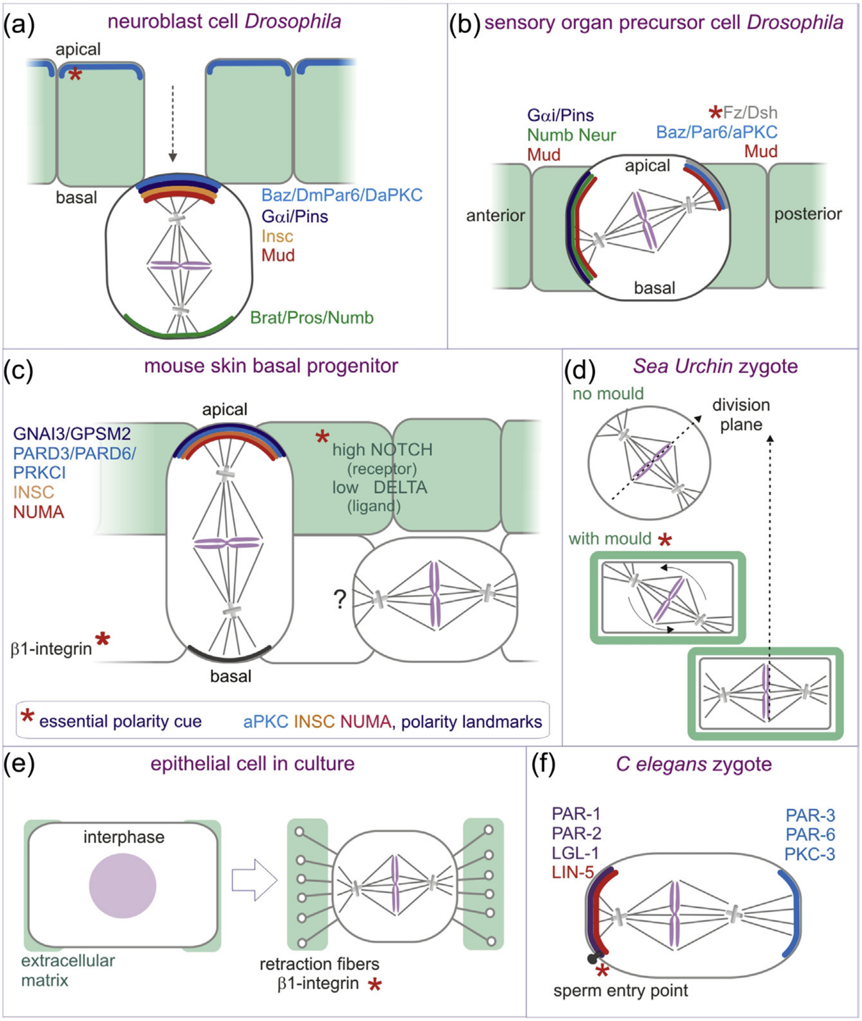


FIGURE 9-14 Diverse polarity cues. (a) A neuroblast arises from the ectodermal layer through a retrieval process (delamination). However, despite a new environment, it maintains its original apical/basal polarity and this serves to orientate the mitotic spindle. Essential cortical landmarks essential for spindle orientation are indicated (Baz, DmPAR6, etc). (b) The sensory organ cell precursor, giving rise to a bristle-carrying sensory organ (Chapter 19, Figure 19-12), divides asymmetrically to give rise to a neuronal and a bristle precursor. Spindle orientation and asymmetric distribution of cellular components occur under the influence of the morphogen Wnt, acting on its receptors Fz/Dsh. Cortical landmarks essential for spindle orientation are indicated. (c) The orientation of the spindle in skin basal-progenitor cells is determined by contact with the basal membrane via β1-integrins and by overall tissue polarity which is represented by high NOTCH expression in the suprabasal cell layer. These polarity cues give rise to a perpendicular asymmetric division. However, occasionally, basal progenitors divide in a planar symmetric fashion, giving rise to two new basal progenitor cells. Essential cortical landmarks that are instrumental in spindle orientation are indicated. (d) In sea urchin fertilized eggs (zygote), the first division can be oriented by placing the egg in a rectangular mold. From this it follows that cell shape may also act as a polarity cue. (e) Focal adhesion contacts also serve as polarity cues. When cells are grown on a matrix with defined contact areas (for instance through precise deposition of fibronectin on a glass substrate), the mitotic spindle aligns with retraction fibers that occur during cell rounding. (f) Sperm entry determines the spindle orientation during the first division in eggs of the nematode *C. elegans*. Again, essential cortical landmarks that are instrumental in spindle orientation are indicated.

cortical cues into spindle orientation and the role played by atypical protein kinase C. We first present the *C. elegans* zygote model to illustrate how numerous components of polarity complexes, including atypical protein kinase C, were discovered through gene mutation and subsequent analysis of phenotypes. We return to (planar) polarity in Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation," where we briefly deal with the orientation of actin-based hairs on wings of *Drosophila*. In Chapter 19, "Cell Fate Determination by Notch," we deal with polarity in the development of bristle-containing sensory organs in the same species.

The PAR proteins in C. *elegans*

The *C. elegans* "par" genes (partitioning defective) were first identified in a genetic screen for maternal-effect mutations resulting in at least three of the following cleavage defects: equal first cleavage, altered orientation of second cleavages, synchronous early cleavages, and abnormalities in localization of P granules (Kemphues et al., 1988). Two of these genes, PAR-3 and PAR-6, encode PDZ-domain proteins that co-localize at the anterior cortex by the end of prophase. At this point, PAR-1, a serine/threonine protein kinase (MARK2) and PAR-2, a ring-finger protein (E3-ligase) (Guo and Kemphues, 1995), localize in a reciprocal manner to the posterior cortex (Figure 9-15). PAR-1 and -2 are connected with LGL-1 (lethal giant larvae) which is a protein that may play a role in the gradual dissolution of the cortical acto-myosin cytoskeleton that occurs at the posterior site (and prevents the attachment of PAR-3 and -6). PAR-4, another kinase (LKB1), and PAR-5, a 14-3-3 protein, are uniformly distributed throughout the cortex and in the cytoplasm. The posterior PAR-3/PAR-6 complex is joined by the atypical PKC-3, and together, by guiding astral microtubules to the posterior site, they regulate the positioning of the mitotic spindle. A similar process occurs at the anterior site (Henrique and Schweisguth, 2003). Anterior and posterior are determined by the site of sperm entry, which operates as a polarity cue.

Atypical protein kinase C, PKC-3 in *C. elegans*, plays an essential role in establishing opposing poles by a mechanism of mutual exclusion of components of polarity complexes. For instance, it phosphorylates and inactivates PAR-1. What this means is that PAR-1 is not operational in the vicinity of PKC-3 and can only contribute to the construction of a polarity complex at a distant side (second pole). Phosphorylation of PAR-2 prevents its association with the cell cortex and thus prevents its association with a polarity complex anywhere near PKC-3. Phosphorylation of LGL-1 prevents its interaction with cortical acto-myosin and it thus

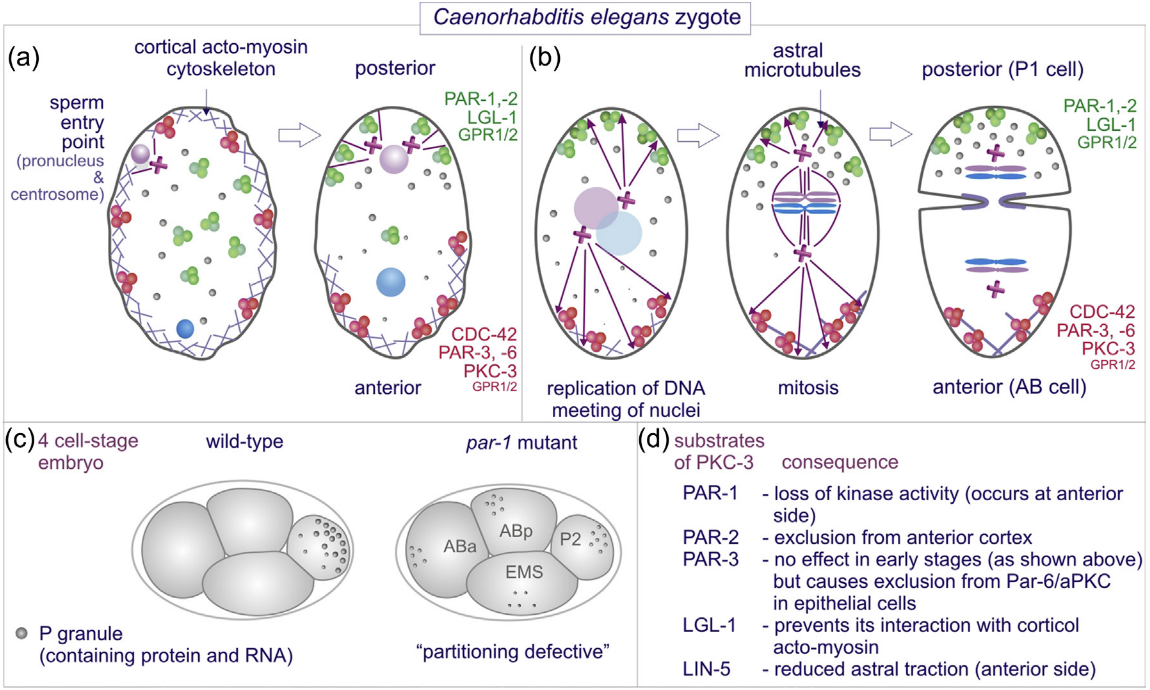

Caenorhabditis elegans zygote
(a)
cortical acto-myosin cytoskeleton
sperm entry point (pronucleus & centrosome)
posterior
PAR-1,-2
LGL-1
GPR1/2
anterior
CDC-42
PAR-3, -6
PKC-3
GPR1/2
(b)
astral microtubules
posterior (P1 cell)
PAR-1,-2
LGL-1
GPR1/2
replication of DNA meeting of nuclei
mitosis
anterior (AB cell)
CDC-42
PAR-3, -6
PKC-3
GPR1/2
(c) 4 cell-stage embryo
wild-type
par-1 mutant
ABa
ABp
P2
EMS
P granule (containing protein and RNA)
"partitioning defective"
(d) substrates of PKC-3
consequence
PAR-1 - loss of kinase activity (occurs at anterior side)
PAR-2 - exclusion from anterior cortex
PAR-3 - no effect in early stages (as shown above) but causes exclusion from Par-6/aPKC in epithelial cells
LGL-1 - prevents its interaction with corticol acto-myosin
LIN-5 - reduced astral traction (anterior side)

FIGURE 9-15 Par proteins in *C. elegans*. (a) The sperm microtubule organizing center arranges the disassembly of the cortical acto-myosin cytoskeleton. This leads to a redistribution of PAR proteins. PAR-3 and PAR-6 remain associated with the cytoskeleton and dominate the future anterior site. PAR-2 and LGL-1 remain "active" at the future posterior site. Each PAR complex has its specific kinase activity, PAR-1 at the posterior and PKC-3 at the anterior site. This leads to phosphorylation of other polarity determining proteins and as a consequence of mutual exclusion resulting in an asymmetric distribution of RNA and proteins. (b) Then, following swelling of the nuclei, DNA replication, condensation of DNA into chromosomes, and dissolution of the nuclear envelope, the astral microtubules are guided by the PAR complexes to ensure the correct orientation of the mitotic spindle. The pulling force of the astral microtubules at the posterior pole exceeds that of the anterior (fewer attachment sites of the dynein complex) resulting in an asymmetric positioning of the metaphase plate. One of the reasons of this is a low presence of GPR-1/2, a protein that connects the cell membrane with motor proteins attached to microtubules, at the anterior side. Once the chromosomes are separated an actin–myosin cleavage furrow bisects the mitotic spindle (cytokinesis). The ensuing asymmetric distribution of cellular components was first visualized by the enrichment of P granules (complexes of RNA and protein) in the posterior (P1) cell. (c) Schematic representation of the four-cell stage of wild-type and a *Par*-1-mutated embryo. Note the difference in partitioning of the P granules. (d) Some of the substrates of PKC-3 and the consequence of their phosphorylation.

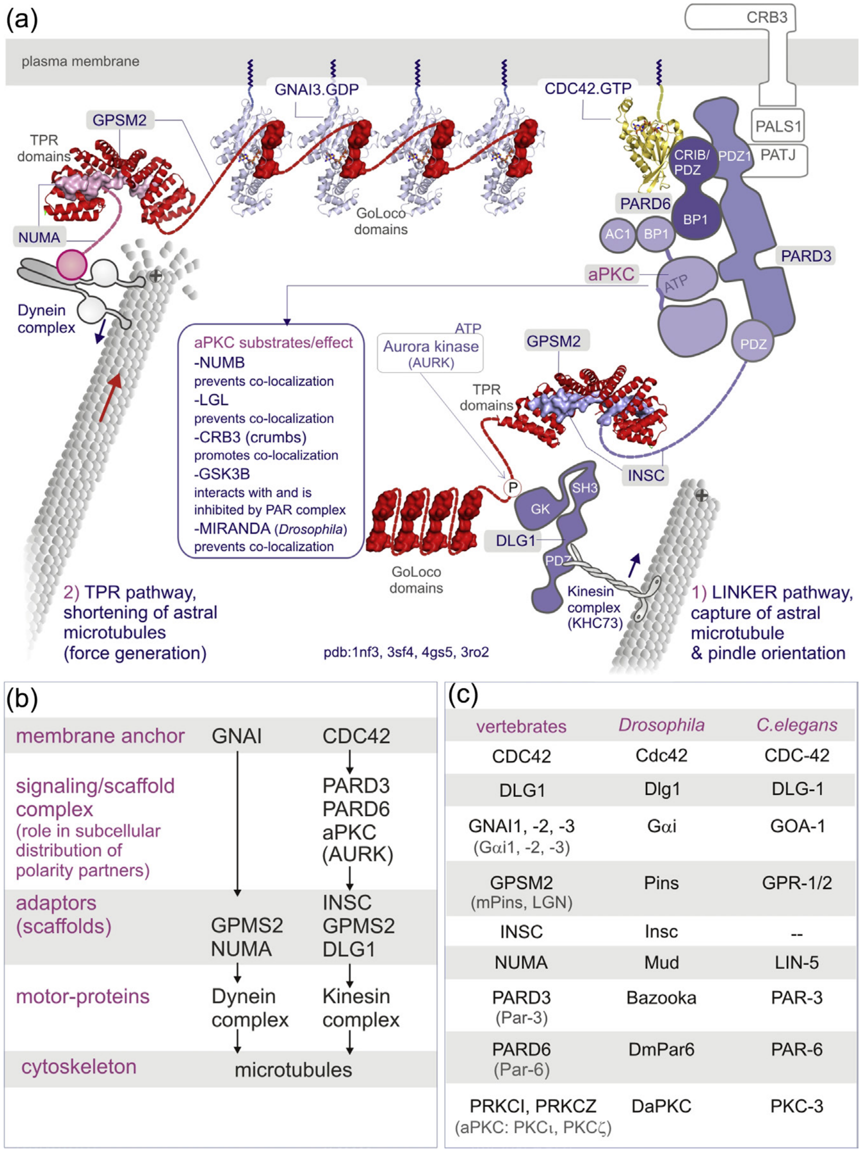
prevents local dissolution of the cytoskeleton with which the PAR-3/PAR-6/PKC-3 complex is connected. In other words, phosphorylation of LGL-1 prevents dislocation of the PKC-3-containing complex (Hao et al., 2006; Munro et al., 2004). In addition, factors are recruited to the different PAR-complexes and these silence mRNA or prevent protein translation, thus giving rise to an altered proteome and transcriptome in the future daughter cells. The accumulation of P granules at the posterior pole witnesses of this asymmetric repartition. An important adaptor protein, GPR-1/2, that connects the cell membrane with motor proteins attached to microtubules (see Figure 9-15) is also unevenly distributed, with more protein at the posterior pole. In connection with this, LIN-5 an adaptor protein associated with GPR-1/2 is a target of PKC-3. Its phosphorylation on numerous serine residues in the C-terminal (S729, S734, S737, S739) weakens the traction force exerted by the astral microtubules (Park and Rose, 2008; Galli et al., 2011). With the anterior astral microtubules being "weaker," the mitotic spindle slides to the posterior pole. Asymmetric spindle positioning and selective retention of cell-fate components cause, after division, the anterior (AB) cell to take on a different destiny from the posterior cell (P1). A first differentiation step is taken in the developing worm.

Web resource

For more information about *C. elegans*, asymmetric cell division and axis formation in the embryo, refer to: http://www.wormbook.org/chapters/www_asymcelldiv/asymcelldiv.html.

Insight into the composition of polarity complexes in flies and mice

Homologs of the *C. elegans* proteins are also regulators of cell polarity in other organisms, ranging from bacteria to vertebrates. In *Drosophila* it was shown that atypical protein kinase C (DaPKC) and PAR proteins play a role in the asymmetric division of type A neuroblasts. These cells are derived from ventral epithelium through a delamination process. The isolated cell maintains its initial polarity and this provides the cue for subsequent positioning of the mitotic spindle (return to Figure 9-14(a)). The apical site contains two apical polarity complexes of which only one involves atypical protein kinase C (Figure 9-16). Similar components have been revealed in mouse skin basal progenitor cells and we will apply mammalian nomenclature in the description



aPKC substrates/effect
- -NUMB prevents co-localization
- -LGL prevents co-localization
- -CRB3 (crumbs) promotes co-localization
- -GSK3B interacts with and is inhibited by PAR complex
- -MIRANDA (*Drosophila*) prevents co-localization

(b)

membrane anchor	GNAI	CDC42
signaling/scaffold complex (role in subcellular distribution of polarity partners)		PARD3 PARD6 aPKC (AURK)
adaptors (scaffolds)	GPMS2 NUMA	INSC GPMS2 DLG1
motor-proteins	Dynein complex	Kinesin complex
cytoskeleton	microtubules	

(c)

vertebrates	*Drosophila*	*C.elegans*
CDC42	Cdc42	CDC-42
DLG1	Dlg1	DLG-1
GNAI1, -2, -3 (Gαi1, -2, -3)	Gαi	GOA-1
GPSM2 (mPins, LGN)	Pins	GPR-1/2
INSC	Insc	--
NUMA	Mud	LIN-5
PARD3 (Par-3)	Bazooka	PAR-3
PARD6 (Par-6)	DmPar6	PAR-6
PRKCI, PRKCZ (aPKC: PKCι, PKCζ)	DaPKC	PKC-3

FIGURE 9-16 Microtubule-attachment sites in a hypothetical mammalian epithelial cell. (a) The composition of the two complexes, named LINKER and TRP, is best described for epithelium-derived neuroblasts in *Drosophila* but corresponding elements have been shown to play a role in spindle orientation in skin progenitor cells in mice (Kulukian and Fuchs, 2013; Williams et al., 2014). For details we refer to the text. Note that connecting the membrane with microtubules requires GPMS2 but employed in two different fashions. Only the first, LINKER, complex contains atypical protein kinase C. Phosphorylation of its substrates mainly prevents co-localization, with the exception of CRB3, and this plays a role in the establishment of an opposite spindle-attachment pole (the "refugees" can only settle elsewhere, after dephosphorylation, out of reach of protein kinase C). (b) Each polarity complex comprises on the one end a membrane anchor and on the other a microtubule attached motor protein. The two are connected by scaffold/adaptor proteins. The assembly downstream of CDC42 also carries a signaling component in the form of the atypical protein kinase C. Aurora kinase (AURK) plays a role in the phosphorylation of the linker region of GPSM2. GRB3 (crumbs) is an apically localized transmembrane protein that, via the adaptor/scaffold protein PALS1 and PATJ, positions PARD6 at the apical region of the cell. (c) Gene names of polarity-complex components in different species.

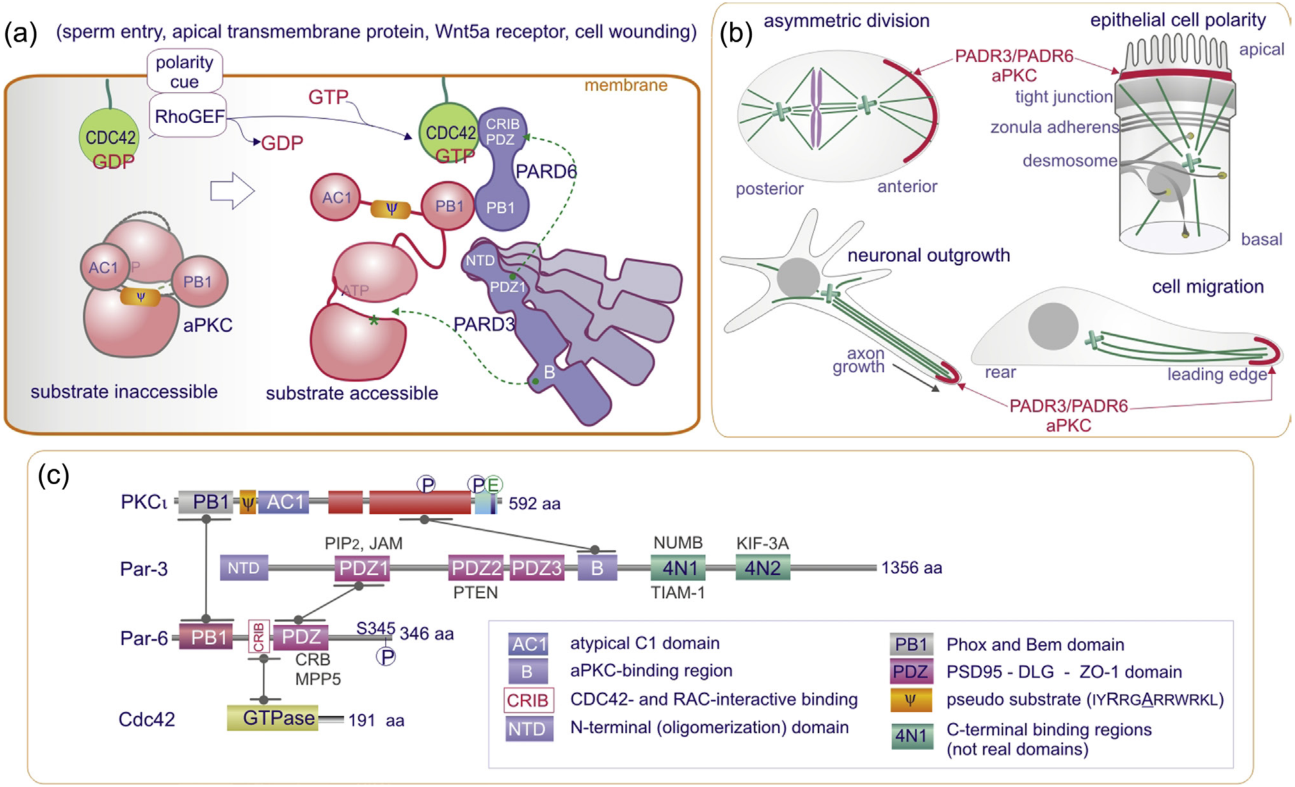


FIGURE 9-17 Proposed model of the activation of atypical protein kinase C by CDC42. (a) Upon the action of a guanine-exchange factor (GEF), CDC42 becomes loaded with GTP and binds the CRIB/PDZ domain of PARD6. This brings about an activation of the atypical protein kinase through removal of the pseudosubstrate and AC1 domain from the catalytic site (removal of the AC1 clamp). The conformational change in the CRIB/PDZ allows PARD6 to interact with the PDZ1 domain of PARD3. This is a big scaffold protein that generally occurs in an oligomeric complex (assembled around the N-terminal domain) (Zhang et al., 2013). PARD3 is a substrate of protein kinase C. (b) Subcellular location of the PARD3/PARD6/aPKC polarity complexes in different conditions and in different cells. (c) Domain architecture and domain interactions of the PARD—atypical protein kinase C polarity complex.

of the polarity complexes. Starting at the membrane, the first complex is anchored by the monomeric GTPase CDC42 in its GTP-bound (active) state. The activation is facilitated by a guanine-exchange factor (GEF) in response to an as yet to be identified activator. CDC42.GTP interacts with PARD6 (Par-6) and through this interaction it modifies the CRIB/PDZ domain which, as a consequence, now binds PARD3 (Par-3) (Peterson et al., 2004). PARD6 also recruits, via its PB1 domain (return to Figure 9-5), atypical protein kinase C (ι or ζ). The transmembrane protein CRB3 (Crumbs) defines the apical positioning of the entire complex. PADR3 binds INSC, an adaptor protein, which in turn binds a second adaptor protein GPSM2 (LGN) (Schober et al., 1999; Wodarz et al., 1999; Yazawa et al., 2011). When phosphorylated by the Aurora protein kinase (AURK), in the linker domain that connects the GoLoco and TPR domains, GPSM2 binds DLG1 and this protein interacts with KHC73, a component of the Kinesin motor–protein complex. As a result of this cascade, the apical membrane is now effectively connected with an astral microtubule. The second complex is anchored to the membrane by GNAI3 (Gαi3), the α-subunit of a heterotrimeric G-protein complex in its GDP-bound (inactive) state. It is bound by the GoLoco domain of the adaptor protein GPSM2 (Jia et al., 2012; Zhu et al., 2011). It is noteworthy that GoLogo competes with Gβγ subunits and with G-protein-coupled receptors for α-subunit binding (return to Chapter 2, “An Introduction to Signal Transduction,” Figure 2-30). The TPR domain of GPSM2 wraps around the second adaptor protein, NUMA, and this in turn associates with the Dynein motor–protein complex. It is generally assumed that through this complex astral microtubules shorten and generate a pulling force on the mitotic spindle (Kulukian and Fuchs, 2013; Bergstralh et al., 2013). The above-described complexes are not always at the same pole, for instance they act at opposite poles in spindle orientation in the sensory organ precursor cell (return to Figure 9-14(b)). It is important to note that under certain circumstances, for instance in columnar epithelial cells of the gut, apical polarity cues such as the tight junction, give rise to a planar positioning of the spindle, that is, astral microtubule attachment is directed toward the basolateral membrane. This means that the polarity complexes migrate from the apical to the basolateral side. In *Drosophila* it has been shown that Bazooka (PAR-3) is phosphorylated by atypical protein kinase C (DaPKC) and causes expulsion from the apical side followed by a reinsertion in the cortical zone of the basolateral membrane (Morais-de-Sa et al., 2010).

Web resource

For more information about the composition of tight junctions and the attached polarity complex go to "tight junction," map hsa04530 (http://www.genome.jp/kegg-bin/show_pathway?hsa04530) of Kyoto Encyclopedia of Genes and Genomes (KEGG).

Review articles

- PAR polarity proteins in *Caenorhabditis elegans*: Hoege, C., Hyman, A.A., 2013. Principles of PAR polarity in *Caenorhabditis elegans* embryos. Nat. Rev. Mol. Cell. Biol. 14, 315–322.
- For a global view of apical-polarity complexes operational in *Drosophila*: Tepass, U., 2012. The apical-polarity protein network in *Drosophila* epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annu. Rev. Cell. Dev. Biol. 28, 655–685.
- For a global view of cell polarity and how it may be involved in cancer:
    - Muthuswamy, S., Xue, B., 2012. Cell polarity as a regulator of cancer cell behavior plasticity. Annu. Rev. Cell. Dev. Biol. 28, 599–625.
- Knoblich, J.A., 2010. Asymmetric cell division: recent developments and their implications for tumor biology. Nat. Rev. Mol. Cell. Biol. 11, 849–860.
- An insightful review about mechanisms of establishing spindle orientation during development: Morin, X., Bellaiche, Y., 2011. Mitotic spindle orientation in asymmetric and symmetric cell divisions during development. Dev. Cell 21, 102–119.
- Spindle orientation in relation to adhesion contacts and mechanical integrity of tissue: Thery, M., Bornens, M., 2006. Cell shape and cell division. Curr. Opin. Cell Biol. 18, 648–657.
- A short but clear review about spindle orientation and epidermal morphogenesis: Kulukian, A., Fuchs, E., 2013. Spindle orientation and epidermal morphogenesis. Phil. Trans. R. Soc. B 368, 20130016.

CDC42 and its role in spindle orientation

How atypical protein kinase C is activated is not yet fully resolved. For certain, the pseudosubstrate and AC1 domain have to be separated from the catalytic domain and this occurs through an interaction with the PB1 domain of PADR6 (Graybill et al., 2012). CDC42 is essential for the activation process, however, what controls its interaction with atypical protein kinase C is still unclear. In the context of cell polarity and spindle orientation, members of the family of RhoGEFs such as MCF2 (DBL, isoform 3), ARHGEF37 (Tuba3), or ARHGEF7 (β-PIX), all are candidates for activating CDC42 (Zihni et al., 2014; Chan and Nance, 2013; Ngok et al., 2014). It then is the role of polarity cues to recruit the appropriate GEF to the same (apical) location as CDC42 (Zihni et al., 2014; Chan and Nance, 2013). The spatial restriction of inositol lipids is an essential determinant in the subcellular localization of CDC42. Polarizing or polarized cells all exhibit segregation of PI-3,4,5-P3 and PI-4,5-P2. In particular, epithelial cells place PI-4,5-P2 at the apical surface and this is sufficient to recruit both CDC42 as well as atypical protein kinase C and PARD6B (Martin-Belmonte et al., 2007). Moreover, PARD3 is also localized at the apical border because it interacts with both the adhesion molecule JAM-1 and with PI-4,5-P2. This cascade of interactions puts atypical protein kinase C, PADR3, and PARD6 at the apical site of the engaging cells, leading to the organization of the tight junction and playing a role in mitotic spindle positioning (Joberty et al., 2000; Ebnet et al., 2004). CDC42 also has a key role in the regulation of the actin cytoskeleton, a process not necessarily linked with activation of atypical protein kinase C, and this too affects cell polarity (Wedlicht-Soldner et al., 2003). Other monomeric GTPases such as Rac1 and RhoA also have a role in the assembly of the cellular junctions and how these junctions connect with the actin cytoskeleton (Ngok et al., 2014; Nishimura et al., 2005; Chen and Macara, 2005).

How does atypical protein kinase C bring about spindle orientation

We have already hinted to the mechanism by which PKC-3 organizes polarity poles in *C. elegans*. Similar processes occur in *Drosophila* and mammalian cells. NUMB is a substrate for atypical protein kinase C and upon phosphorylation it is destined to leave the apical site and contributes to the formation of an opposite spindle (Smith et al., 2007). The same applies for LGL (Tian and Deng, 2008). On the contrary, phosphorylation of CRB3 reinforces its interaction with the PARD6-polarity complex and this may serve as a feed-forward mechanism. The protein kinase GSK3B is inhibited through an interaction with the PARD-atypical protein kinase C complex. In addition, it is phosphorylated but the importance of this is not clear. Bazooka is a substrate of DaPKC and its phosphorylation causes apical exclusion. This contributes to photoreceptor polarity remodeling (Walther and Pichaud, 2010). Lastly, the *Drosophila* membrane-bound protein Miranda is also phosphorylated and this too prevents co-localization. As a result it can only settle, after dephosphorylation, in the membrane cortical zone at a distance from active protein kinase C (Atwood and Prehoda, 2009). In the *Drosophila* neuroblast, Miranda and Numb localize at the basal site where they cause the repression of genes involved in cell-cycle regulation. As a result they contribute to asymmetric cell division giving rise to an "apical" proliferative stem cell and a "basal" committed progenitor cell known as ganglion mother cell.

MAMMALIAN PROTEINS THAT TAKE UP THE FUNCTION OF MIRANDA

There is no known mammalian homolog of Miranda. Asymmetric localization of mammalian Prox1, Staufen, and Trim3, which are established Miranda cargos in *Drosophila*, seem to be taken up by other proteins such as the proteoglycan NG2 (CSPG4), the pumilio homolog-2 (PUM2), or RNA helicase DDX1. NG2 segregates EGF receptors in oligodendrocyte precursor cells (Sugiarto et al., 2011) and its loss coincides with premalignant abnormal self-renewal rather than differentiation of oligodendrocyte progenitors (improper partitioning of cell-cycle regulators). PUM2 and DDX1 are the apically located RNA-granule proteins that interact with and sequester the RNA-binding proteins Prox1 and Staufen (Vessey et al., 2012).

The importance of spindle orientation in tissue development

In two examples we show the importance of polarity complexes in spindle orientation and subsequent tissue development. We start with the developing skin of mice (in utero). The basal layer is composed of semi-stem cells, named basal-progenitor cells, which arise from true stem-cell compartments that are found in hair follicles. The majority of these cells orients its mitotic spindle perpendicular to the basement membrane, giving rise to one progenitor and one supra-basal cell. The latter cells undergo a number of phenotypic changes and finally end up as keratin-packed dead cells that constitute the stratum corneum. The ensemble of layers qualifies as a stratified and keratinized squamous epithelium (like the human skin) (Figure 9-18). As an exception to the rule, a few basal progenitor cells exhibit planar spindles and their daughters remain skin progenitor cells (symmetric division). With the loss of GPSM2, present in both polarity complexes shown in Figure 9-16, or with the loss of INSC (only present in complex 1) and GNAI3 (only present in complex 2), all cells show planar spindle orientation. As a result the skin completely loses its stratified characteristics. Loss of only INSC or PARD3 or GNAI3 gives rise to an intermediate state, a poorly developed stratified squamous epithelium. We still have only a few minor hints of how spindles position in lateral division and how the choice to switch to a perpendicular spindle orientation is made. Loss of both polarity complexes gives rise to planar spindle orientation suggesting that this might be a default pathway with its own, yet unknown, positional cues (Williams et al., 2011, 2014). Cadherins, found in both the zonula adherens and desmosomes (see Chapter 11, “Signal Transduction to and from Adhesion Molecules,”Figure 11-10), have been shown to act as a possible polarity cue in the maintenance of a simple cubical kidney epithelium in culture (den Elzen et al., 2009).

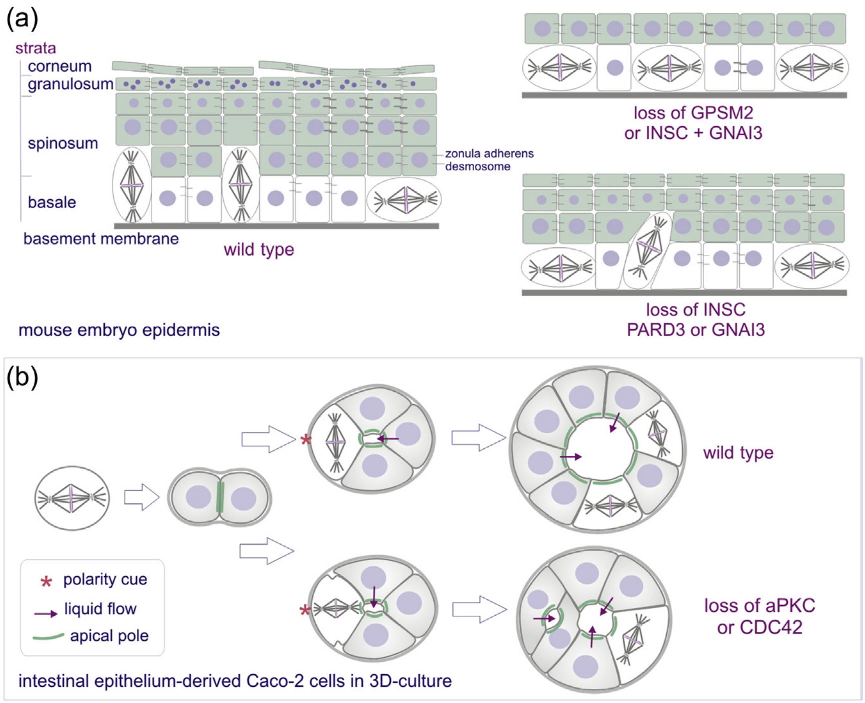


FIGURE 9-18 Consequences of silencing of polarity complex components. (a) In the basal layer of cells in the mouse skin, named basal progenitor cells, the majority of the spindles orientate perpendicular to the basal membrane, only very few cells exhibit a planar spindle orientation. Both orientations are vital for the elaboration of a stratified and keratinized squamous epithelium. Loss of GPSM2 or INSC + GNAI3 results in a "default" planar spindle orientation with the consequence of a total loss of the differentiation characteristics of the epithelium. Loss of only INSC, PARD3, or GNAI3 gives rise to an intermediate state, a poorly developed stratified squamous epithelium (Williams et al., 2014). (b) Human Caucasian colon adenocarcinoma cells, Caco-2 cells, form cysts when grown in Matrigel. Integrin binding to the basement membrane acts as a polarity cue and all cells exhibit a perpendicular spindle orientation (relative to basement membrane). At the four-cell stage Cl^- and Na^+ ions enter the extracellular space at the apical site, followed by water, resulting in the formation of a liquid-filled cavity (cyst). When CDC42 or atypical protein kinase C is lacking, the spindle orientation becomes erratic and multiple little cavities are formed.

The other example concerns cyst formation of intestinal epithelial cells, named Caco-2, kept in culture but being embedded in Matrigel (a mixture of extracellular matrix components). After two divisions, the cells start to create a small liquid-filled cavity caused by transport of ions across the apical membrane (Cl^- and Na^+). Subsequent divisions are guided by the basement membrane/integrin polarity cue (Myllymaki et al., 2011) giving rise to a growing cyst (Figure 9-18). With the loss of CDC42 or PKCζ, spindle orientation becomes erratic and multiple liquid cavities develop (Jaffe et al., 2008; Durgan et al., 2011). A similar finding is described in the developing mouse pancreas, where loss of CDC42 causes the loss of tube formation and instead the developing gland displays numerous small cavities without any organization (Kesavan et al., 2009).

ATYPICAL PROTEIN KINASE C IN CELL MIGRATION AND AXONAL OUTGROWTH

Beyond its roles in the determination of asymmetric division during development and in the organization of epithelial sheets, polarity also provides directionality to migrating cells and determines the site of axon outgrowth.

Protein kinase C and migration of astrocytes

Astrocytes are the major source of glial cells in the brain. They operate in the differentiation and functioning of neurons, not only as supporting structures but also in the regulation of synaptic transmission and thus the organization of neuronal circuitry. When a scratch is made in a near-confluent culture of astrocytes, the surrounding cells present new tips that grow into the empty space.

ASTROGLIAL CELLS IN EINSTEIN'S BRAIN

Although his body was cremated, Albert Einstein's brain was preserved for pathologists and posterity. A scientific report has indicated that his cerebral cortex contained a considerable excess of astrocytic tissue (in comparison with four others) (Colombo et al., 2006). Of course, this news was promptly reported in the Sunday papers (Dobson, 2006) but the authors of the original report wisely claimed no particular significance for their observation in terms of the great man's cognitive ability.

At the site of the scratch, the astrocytes accumulate Scrib and βPIX (Osmani et al., 2006) guanine-exchange proteins that cause the activation of CDC42. From here two parallel polarizing processes are set in train. Firstly, CDC42 orchestrates the formation of a membrane protrusion through activation of RAC1 which organizes the actin fibers into a gel-like network. The recruitment and activation of RAC1 again requires βPIX (Ten Klooster et al., 2006). Secondly, CDC42 reorients the centrosome-attached microtubule network perpendicular

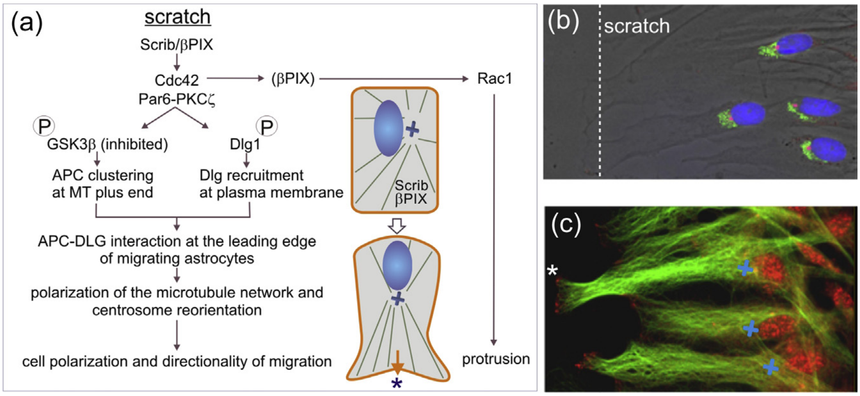

(a)
scratch
Scrib/βPIX
Cdc42
(βPIX)
Rac1
Par6-PKCζ
P
GSK3β (inhibited)
P
Dlg1
APC clustering at MT plus end
Dlg recruitment at plasma membrane
Scrib βPIX
APC-DLG interaction at the leading edge of migrating astrocytes
polarization of the microtubule network and centrosome reorientation
cell polarization and directionality of migration
protrusion
*
(b)
scratch
(c)
*

FIGURE 9-19 Protein kinase C and migration of astrocytes. (a) A scratch in near confluent culture of astrocytes provokes reorientation and migration of the adjacent cells to fill the gap. This is guided by the accumulation of the scaffold protein Scrib, bound to βPIX (RhoGEF, ARHGEF7). The ensuing activation of the Par-6 polarity complex causes protrusion formation, implying the GTPase Rac1, and to reorientation of the microtubule cytoskeleton. In order to achieve this, atypical PKCζ phosphorylates and inactivates GSK3β, causing clustering of APC at the microtubule plus ends, and it phosphorylates and activates Dlg, which is now recruited at the membrane. Binding of Dlg and APC assures anchorage of the microtubules to the protruding membrane to provide directionality to the migration process. (b) Immunocytochemical staining of the Golgi apparatus (green), the centrosome (red), and the nucleus (blue) reveals the orientation of the astrocytes perpendicular to the scratch line. (c) Immunocytochemical staining of APC (red and highlighted by asterisk) at the plus end of microtubules (green). The centromeres (blue cross) have been added to show the relative position in relation to the nucleus (red). *(Images b and c courtesy of Dr. Etienne-Manneville, Insitut Pasteur, Paris, France.)*

to the direction of the scratch and the Golgi apparatus is directed perpendicular to the newly formed microtubule axis (Figure 9-19). Here, atypical protein kinase C, complexed with PARD3 and PARD6, directly interacts with and inhibits GSK3β (phosphorylation is not necessary) (Hur and Zhou, 2010) so allowing dephosphorylation of the adenomatous polyposis coli (APC) protein (see Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation"). This now binds to the plus ends of centromere-attached microtubules. Interestingly, APC also recruits mRNA through binding to their 3′-untranslated region (3′-UTR) and among the targets is β2B tubulin, a component of the microtubules (composed of type α- and β-tubulin). As a consequence, β2B tubulin is synthesized preferentially at the plus ends of dynamic microtubules such as that are found in the growth cone. APC serves as a platform binding functionally related protein and RNA networks, allowing the self-organizing of microtubule polymerization (Preitner et al., 2014). Note that microtubules are composed of dimers of two subunits, α- and β-tubulin. Binding of the mRNA of α1A tubulin has not yet been reported (see also Figures 9-20 and 9-22).

The second substrate of protein kinase C is DLG1 which on phosphorylation localizes to the plasma membrane. Interaction between DLG1 and APC, in addition to the action of tubulin-bound motor proteins such as dynein, enables the microtubules to reorient the centromere. CDC42-mediated activation of atypical protein kinase C thus provides both the means to migrate (protrusion formation) and the necessary directionality for the migrating cell (reorientation of centrosome-attached microtubules). The domain architecture of the components involved in astrocyte migration is shown in (Figure 9-20).

Conventional protein kinase C too plays a role in cell migration. For instance, in human umbilical vein endothelial cells. In response to chemotactic stimuli, such as PDGF or interleukin-8 (IL8), phospholipase C is activated and this results in the production of diacylglycerol and a rise in intracellular free Ca^{2+}. The ensuing activation of protein kinase C-α (PRKCA) leads to the phosphorylation of RhoGDI (ARFGDIA) and this causes the release of monomeric GTPases RHOA and RAC1 from their inhibitor. Once they are loaded with GTP, RHOA sensitizes smooth muscle myosin for Ca^{2+}-mediated activation, through inhibition of the opposing phosphatase, and RAC1 stimulates actin filament formation at the

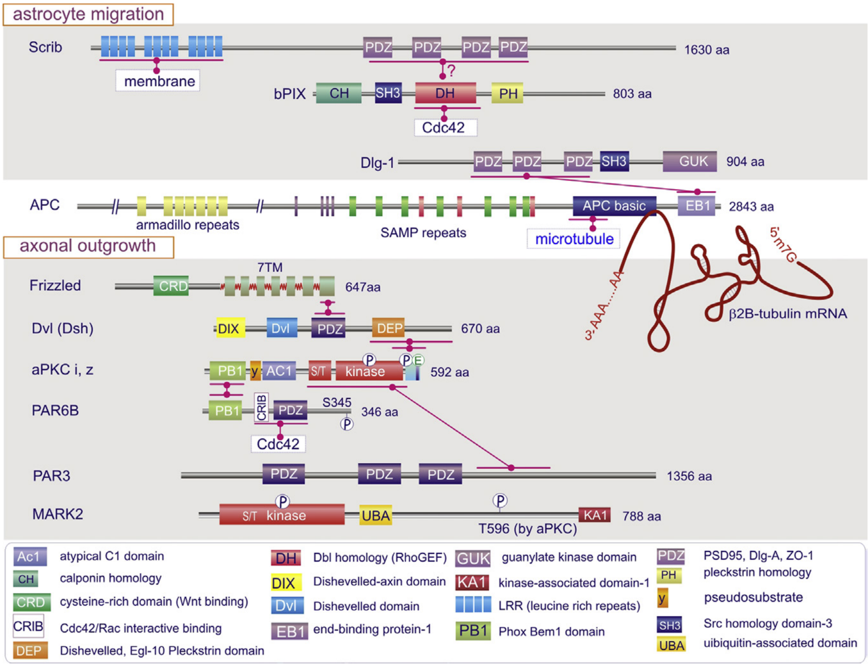


FIGURE 9-20 Domain structure of proteins involved in astrocyte migration and axonal outgrowth. Domain interactions are indicated by red lines. The basic region of APC binds the 3′-untranslated region (3′-UTR) of mRNA (or it binds an mRNA-binding protein). APC is shared between astrocyte migration and axonal outgrowth.

leading edge (Tsai et al., 2014). For full information return to Chapter 6, "Intracellular Calcium," section "connecting Ca^{2+} waves with cytoskeletal activity" (summarized in Figure 6-23).

Protein kinase C and axonal outgrowth

Axons are typically long and thin, of uniform width, branching at right angles from the cell body (Figure 9-21). Dendrites are relatively short and as they emerge from the cell body they appear thick, but become thinner with increasing distance and then undergo Y-shaped branching. These two cellular structures, with the synaptic contacts they make with other cells, are fundamental for neuronal function enabling them to receive and transmit signals. Axon formation in cultured hippocampal neurons is extensively studied. These cells initially form multiple neurites which expand and retract, until, at a moment, just one of them stretches a little beyond the others to become the unique axon. Its formation prevents the extension of other neurites, most likely through depletion of Cdc42 and these cells become dendrites.

The tip of the growing axon accumulates disheveled (DVL), associated with Frizzled (FZD), a receptor for the family of WNT ligands (see Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation"). Both DVL and FZD were discovered in *Drosophila* mutants in which the epithelial cells had lost the polarized positioning of the wing hairs (Figure 12-3). DVL binds the atypical protein kinase C/PARD6/PARD3 polarity complex and this leads to stabilization and activation (Figure 9-22). Although CDC42 also accumulates in the growing axon, its precise role in the activation of DVL-associated PKC remains to be determined (Schwamborn and Puschel, 2004). As CDC42 is capable of stimulating the RAC1 guanine–nucleotide exchange factor STEF, which accumulates at the tip of the growing axon, it certainly plays a role in the local organization of the actin cytoskeleton (not shown) (Nishimura et al., 2005).

An important substrate of the atypical protein kinase C is the serine/threonine protein kinase MARK2 (also known as PAR-1 in *C. elegans*) which, on phosphorylation is inactivated and detaches from the membrane (Hurov et al., 2004). This removes an inhibitory constraint for a number of components that normally interact with microtubules. Among these are MAPT and MAP1B. The other target is GSK3β which upon interaction with the PAR–protein kinase C complex becomes inactivated, so enabling the association of APC and with microtubules. GSK3β is phosphorylated but its significance remains unclear. These proteins stabilize microtubules and also facilitate their attachment to the plasma membrane. In so doing, they provide important support for the growing axon. Again, APC carries the pRNA for β2b tubulin, thus facilitating microtubule growth at the plus end. The domain architecture of proteins involved in axon outgrowth is summarized in Figure 9-20.

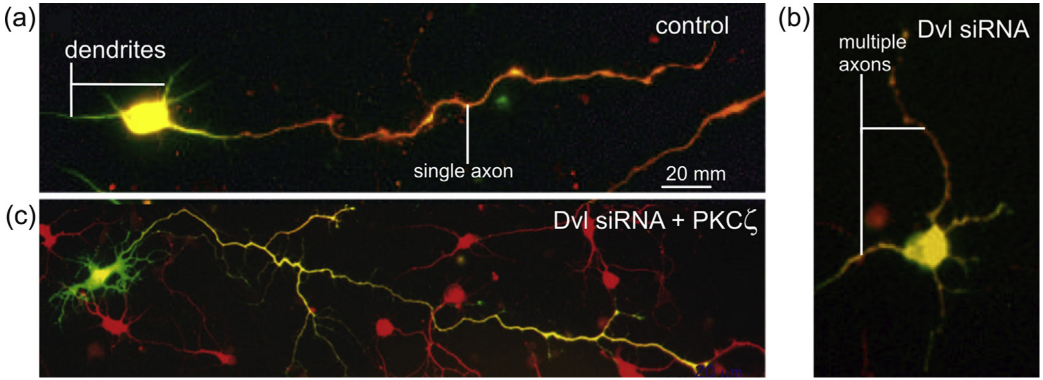


FIGURE 9-21 Axon-outgrowth. (a) Morphology of hippocampal neurons in culture. Note the numerous short dendrites and the single long axon. (b) Loss of expression of Dvl, through addition of siRNA, leads to the loss of polarity, numerous short axons are produced. (c) Injection of PKCζ into Dvl-depleted neurons restores the polarity with the formation of a single long axon. *Adapted from Arimura and Kaibuchi (2007)*.

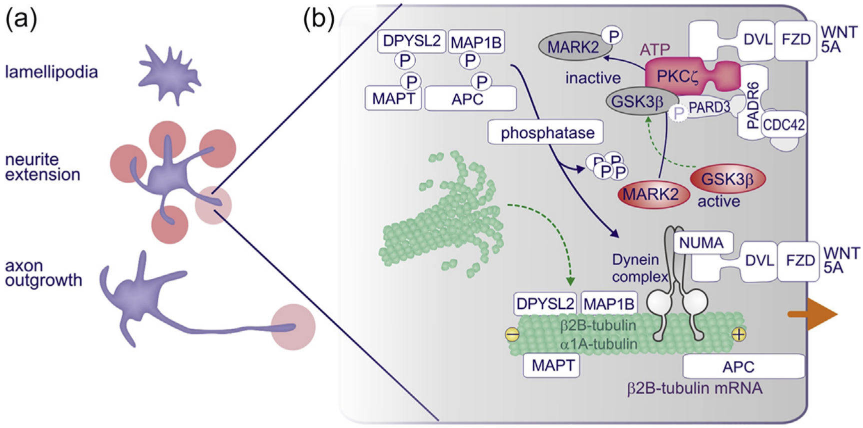


FIGURE 9-22 Composition of a polarity complex in axonal outgrowth. (a) Hippocampal neurons initially form multiple neurite extentions, which expand and retract, until, in a moment, just one stretches a little further and becomes the unique axon (negative feedback toward the many other extensions). (b) Axon outgrowth, stimulated by WNT5A at the Frizzled (FZD) receptor is initiated by the binding of an atypical PKCζ/PARD3/PARD6 polarity complex due to Disheveled (DVL). CDC42 is also present in the complex but its role in mediating activation of PKCζ in this particular context is unclear. However, it does play a role in the activation of STEF, a Rac1 GTPase that accumulates in the tip of the growing axon, and this will lead to local actin polymerization (Nishimura et al., 2005). Activated PKCζ leads to phosphorylation and inactivation of MARK2. The protein kinase GSK3β is also phosphorylated but its inactivation is entirely dependent on binding to the polarity complex. Loss of kinase activity leads to dephosphorylation of the microtubule-binding proteins DPYSL2 (CRMP2), MAP1B, MAPT (Tau), and APC (adenomatous polyposis coli). These now bind to microtubules, thereby enhancing their stability (preventing depolymerization) and their interaction with the plasma membrane (to provide directionality to the axon outgrowth). APC also brings β2B tubulin mRNA to the microtubules and this may play a role in sustained production of tubulins at the site of microtubule assembly (plus end) (Preitner et al., 2014). DVL also interacts with NUMA (through its DEP domain), and this allows attachment of the Dynein motor–protein complex (Lu and Johnston, 2013).

References

Adams, D.R., Ron, D., Kiely, P.A., 2011. RACK1, a multifaceted scaffolding protein: structure and function. Cell Commun. Signal 9, 22.

Ailles, L.E., Weissman, I.L., 2007. Cancer stem cells in solid tumours. Curr. Opin. Biotechnol. 18, 460–466.

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., Kaibuchi, K., 1996. Identification of a putative target for Rho as the serine/threonine kinase protein kinase N. Science 271, 648–650.

Angel, P., Imagawa, M., Chi, R., et al., 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49, 729–739.

Arimura, N., Kaibuchi, K., 2007. Neuronal polarity: from extracellular signals to intracellular mechanisms. Nat. Rev. Neurosci. 8, 194–205.

Asselin-Labat, M.-L., Vaillant, F., Sheridan, J.M., Pal, B., Wu, D., Simpson, E.R., Yasuda, H., Smyth, G.K., Martin, T.J., Lindeman, G.J., Visvader, J.E., 2010. Control of mammary stem cell function by steroid hormone signalling. Nature 465, 798–802.

Atwood, S.X., Prehoda, K.E., 2009. aPKC phosphorylates Miranda to polarize fate determinants during neuroblast asymmetric cell division. Curr. Biol. 12, 723–729.

Baan, B., Pardali, E., ten Dijke, P., van Dam, H., 2010. In situ proximity ligation detection of c-Jun/AP-1 dimers reveals increased levels of c-Jun/Fra1 complexes in aggressive breast cancer cell lines in vitro and in vivo. Mol. Cell Proteomics 9, 1982–1990.

Baud, V., Karin, M., 2001. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol. 11, 327–377.

Behrens, A., Jochum, W., Sibilia, M., Wagner, E.F., 2000. Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. Oncogene 19, 2657–2663.

Benes, C.H., Wu, N., Elia, A.E., Dharia, T., Canntley, L.C., Soltoff, S.P., 2005. The C2 domain of PKCδ is a phosphotyrosine binding domain. Cell 121, 271–280.

Berenblum, I., 1941. Cocarcinogenic action of croton resin. Cancer Res. 1, 48.

Bergstralh, D.T., Haack, T., St Johnston, D., 2013. Epithelial polarity and spindle orientation: intersecting pathways. Phil. Trans. R. Soc. B 368, 20130291.

Berns, H., Human, R., Hengerer, B., Kiefer, F.N., Battegay, E.J., 2000. RACK1 is up-regulated in angiogenesis and human carcinomas. FASEB J. 14, 2549–2558.

Berridge, M.J., Irvine, R., 2000. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315–321.

Besson, A., Yong, V.W., 2000. Involvement of p21(Waf1/Cip1) in protein kinase C α-induced cell cycle progression. Mold. Cell Biol. 20, 4580–4590.

Boehm, J., Kang, M.G., Johnson, R.C., Esteban, J., Huganir, R.L., Malinow, R., 2006. Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. Neuron 51, 213–225.

Bohmann, D., Tjian, R., 1989. Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-Jun. Cell 59, 709–717.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., Tjian, R., 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238, 1386–1392.

Bonnet, D., Dick, J.E., 1997. Human acute myeloid leukemia is organized as a hierarch that originates from a primitive hematopoietic cell. Nat. Med. 3, 730–737.

Borner, C., Ueffing, M., Jaken, S, Parker, P.J., Weinstein, I.B., 1995. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. J. Biol. Chem. 270, 78–86.

Boyle, W.J., Smeal, T., Defize, L.H., et al., 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 64, 573–584.

Buday, L., Downward, J., 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73, 611–620.

Cameron, A.J.M., Escribano, C., Saurin, A.T., Kostelecky, B., Parker, P.J., 2009. PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity. Nat. Struct. Mol. Biol. 16, 624–630.

Campbell, K.H.S., McWhir, J., Ritchie, W.A., Wilmut, I., 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380, 64–66.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, Y., Nishizuka, Y., 1982. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847–7851.

Chan, E., Nance, J., 2013. Mechanisms of Cdc42 activation during contact-induced cell polarization. J. Cell Sci. 126, 1692–1702.

Chen, X., Macara, I.G., 2005. Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. Nat. Cell Biol. 7, 262–269.

Collingridge, G.L., John, T.R., Isaac, J.T.R., Wang, Y.T., 2004. Receptor trafficking and synaptic plasticity. Nat. Rev. Neurosci. 5, 952–962.

Collins, B.J., Deak, M., Murray-Tait, V., Storey, K.G., Alessi, D.R., 2005. In vivo role of the phosphate groove of PKD1 defined by knockin mutation. J. Cell Sci. 118, 5023–5034.

Colombo, J.A., Reisin, H.D., Miguel-Hildago, J.J., Rajkowska, G., 2006. Cerebral cortex astroglia and the brain of a genius: a propos de A. Einstein's. Brain Res. Rev. 52, 257–263.

Colon-Gonzales, F., Kazanietz, M.G., 2006. C1 domain exposed: from diacyglycerol binding to protein-protein interactions. Biochim. Biophys. Acta 1761, 827–837.

Cook, J., Hewett, C., Hieger, I., 1933. The isolation of a cancer-producing hydrocarbon from coal tar. J. Chem. Soc. 395–405.

Corbit, K.C., Trakul, N., Eves, E.M., Diaz, B., Marshal, M., Rosner, M.R., 2003. Activation of RAF1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J. Biol. Chem. 278, 13061–13068.

Coussens, L., Parker, P.J., Rhee, L., et al., 1986. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233, 859–866.

Crombie, L., Games, M.L., Pointer, D.J., 1968. Chemistry and structure of phorbol, the diterpene parent of the co-carcinogen of croton oil. J. Chem. Soc. (Perkin1) 11, 1362.

Csukai, M., Chen, C.H., De Matteis, M.A., Mochly-Rosen, D., 1997. The coatamer protein β-COP, a selective binding protein (RACK) for protein kinase Cε. J. Biol. Chem. 272, 29200–29206.

den Elzen, N., Buttery, C.V., Maddugoda, M.P., Ren, G., Yap, A.S., 2009. Cadherin adhesion receptors orient the mitotic spindle during symmetric cell division in mammalian epithelia. Mol. Biol. Cell 20, 3740–3750.

Derijard, B., Hibi, M., Wu, I.H., et al., 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76, 1025–1037.

Ding, X.W., Wu, J.H., Jiang, C.P., 2010. ABCG2: a potential marker of stem cells and noel target in stem cell and cancer therapy. Life Sci. 86, 613–637.

Dobson, R., 2006. Inside Einstein's Brain. Independent on Sunday. 4.

Dujardin, D.L., Vallee, R.B., 2002. Dynein at the cortex. Curr. Opin. Cell Biol. 14, 44–49.

Dujon, B., 2005. Comment Évoluent Nos Genes. Edition le Pommier.

Durgan, J., Kaji, N., Jin, D., Hall, A., 2011. Par6B and atypical PKC regulate mitotic spindle orientation during epithelial morphogenesis. J. Biol. Chem. 286, 12461–12474.

Ebnet, K., Susuki, A., Ohno, S., Vestweber, D., 2004. Junctional adhesion molecules (JAMs): more molecules with dual functions? J. Cell Sci. 117, 19–29.

Eferl, R., Wagner, E.F., 2003. AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer 3, 859–868.

Facchinetti, V., Ouyang, W., Wei, H., Lazorchak, A., Goold, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q., Sessa, W.C., Qin, J., Zhang, O., Su, B., Jacinto, E., 2008. The mammalian target off rapamycin complex 2 controls folding and stability of Akt and proteinkinase C. EMBO J. 27, 1932–1943.

Feng, J., Cai, X., Zhao, J., Yan, Z., 2001. Serotonin receptors modulate GABA(A) receptor channels through activation of anchored protein kinase C in prefrontal cortical neurons. J. Neurosci. 21, 6502–6511.

Fukomoto, S., Nishizawa, Y., Hosoi, M., et al., 1997. Protein kinase Cd inhibits the proliferation of vascular smooth muscle cells by suppressing G1 cyclin expression. J. Biol. Chem. 272, 13816–13822.

Galli, M., Munoz, J., Portegijs, V., Boxem, M., Gill, S.W., Heck, A.J., van den Heuvel, S., 2011. A PKC phosphorylates NuMA-related LIN-5 to position the mitotic spindle during asymmetric division. Nat. Cell Biol. 13, 1132–1138.

Geijsen, N., Spaargaren, M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., Coffer, P.J., 1999. Association of RACK1 and PKCb with the common b-chain of the IL-5/IL3/GM-CSF receptor. Oncogene 18, 5126–5130.

Gille, H., Downward, J., 1999. Multiple ras-effector pathways contribute to G(1) cell cycle progression. J. Biol. Chem. 274, 22033–22040.

Goode, N., Hughes, K., Woodgett, J.R., Parker, P.J., 1992. Differential regulation of glycogen synthase kinase-3b by protein kinase C isotypes. J. Biol. Chem. 267, 16878–16882.

Goodnight, J.A., Mischak, H., Kolch, W., Mushinsky, J.F., 1995. Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. J. Biol. Chem. 270, 9991–10001.

Graybill, C., Wee, B., Atwood, S.X., Prehoda, K.E., 2012. Partitioning-defective protein 6 –Par-6) activates atypical protein kinase C (aPKC) by pseudosubstrate displacement. J. Biol. Chem. 287, 21003–21011.

Grivennikov, S., Karin, M., 2008. Autocrine IL-6 signaling: a key event in tumorigenesis? Cancer Cell 13, 7–9.

Guo, S., Kemphues, K.J., 1995. Par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a ptuative ser/thr kinase that is asymmetrical distributed. Cell 81, 611–620.

Guo, Y., Wang, W., Wang, J., Feng, J., Wang, Q., Jin, L., 2013. Receptor for activated C-kinase 1 promotes hepatocellular carcinoma growth by enhancing mitogen-activated protein kinase kinase 7 activity. Hepatology 57, 140–151.

Gurdon, J.B., 1962. Adult frogs derived from the nuclei of single somatic cells. Dev. Biol. 256–273.

Hannun, Y.A., Obeid, L.M., 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. 9, 139–150.

Hao, Y., Boyd, L., Seyoux, G., 2006. Stabilization of cell polarity by the *C. elegans* RING protein PAR-2. Dev. Cell 10, 199–208.

Henrique, D., Schweisguth, F., 2003. Cell polarity; the ups and downs of the Par6/aPKC complex. Curr. Opin. Genet. Dev. 13, 341–350.

Hornbeck, P.V., Kornhauser, J.M., Tkachev, S., Zhang, B., Skrzypek, E., Murray, B., Latham, V., Sullivan, M., 2012. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res. 40, D261–D270.

House, C., Kemp, B.E., 1987. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. Science 238, 1726–1728.

Housey, G.M., Johson, M.D., Hsiao, W.L., et al., 1998. Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell 52, 343–345.

Hsiao, W.M., Housey, G.M., Johnson, M.D., Weinstein, I.B., 1989. Cell that overproduce protein kinase C are more susceptible to transformation by an activated H-ras oncogene. Mol. Cell Biol. 9, 2641–2647.

Hur, E.-M., Zhou, F.-Q., 2010. GSK3 signaling in neural development. Nat. Rev. Neurosci. 11, 539–551.

Hurley, J.H., Newton, A.C., Parker, P.J., Blumberg, P.M., Nishizuka, Y., 1997. Taxonomy and function of C1 protein kinase C homology domains. Protein Sci. 2, 477–480.

Hurov, J.B., Watkins, J.M., Piwnica-Worms, H., 2004. Atypical protein kinase C phosphorylates PAR-1 kinases to regulate localization and activity. Curr. Biol. 14, 736–741.

Iliopoulos, D., Hirsch, H.A., Struhl, K., 2009. An epigenetic switch involving NF-κB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell 139, 693–706.

Imbra, R.J., Karin, M., 1986. Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. Nature 323, 555–558.

Inoue, M., Kishimoto, A., Takai, Y., Nishizuka, Y., 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent proteases from rat brain. J. Biol. Chem. 252, 7610–7616.

Jaffe, A.B., Kaji, N., Durgan, J., Hall, A., 2008. Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. J. Cell Biol. 183, 625–633.

Janoff, A., Klassen, A., Troll, W., 2000. Local vascular changes induced by the co-carcinogen, phorbol myristate acetate. Cancer Res. 30, 2568–2571.

Jia, M., Li, J., Zhu, J., Wen, W., Zhang, M., Wang, W., 2012. Crystal structures of the scaffolding protein LGN reveal the general mechanism by which GoLoco binding motifs inhibit the release of GDP from Galphi subunit in G-coupled heterotrimeric proteins. J. Biol. Chem. 287, 36766–36776.

Joberty, G., Petersen, C., Gao, L., Macara, I.G., 2000. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat. Cell Biol. 2, 531–539.

Kakumoto, K., Sasai, K., Sukezane, T., Oneyama, C., Ishimaru, S., Shibutani, K., Mizushima, H., Mekada, E., Hanafusa, H., Akagi, T., 2006. FRA1 is a determinant for the difference in RAS-induced transformation between human and rat fibroblasts. Proc. Natl. Acad. Sci. USA. 103, 5490–5495.

Kallunki, T., Deng, T., Hibi, M., Karin, M., 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 87, 929–939.

Kappelmann, M., Bosserhof, A., Kuhal, S., 2014. AP-1/c-Jun transcription factors; regulation and function in malignant melanoma. Eur. J. Cell Biol. 93, 76–81.

Karin, M., Liu, Z., Zandi, E., 1997. AP-1 function and regulation. Cell 9, 240–246.

Katakami, Y., Kaibuchi, K., Sawamura, M., Takai, Y., Nishizuka, Y., 1984. Synergistic action of protein kinase C and calcium for histomain release from rat peritoneal mast cells. Biochem. Biophys. Res. Commun. 121, 573–578.

Kavanaugh, A.F., Lightfoot, E., Lipsky, P.E., Oppenheimer-Marks, N., 1991. Role of CD11:CD18b in adhesion and transendothelial migration of T cells. Analysis utilizing CD18-deficient T cell clones. J. Immunol. 146, 4149–4156.

Kemphues, L.J., Priess, J.R., Morton, D.G., Cheng, N., 1988. Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. Cell 52, 311–320.

Kesavan, G., Wolfhagen, S., Greiner, T.U., Johansson, J.K., Kobberup, S., Wu, X., Brakebusch, C., Semb, H., 2009. Cdc42-mediated tubelogenesis controls cell specification. Cell 139, 791–801.

Kheifets, V., Mochly-Rosen, D., 2007. Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. Pharmacol. Res. 55, 467–476.

Kieser, A., Sietz, T., Adler, H.S., et al., 1996. Protein kinase Cz reverts v-raf transformation of NIH-3T3 cells. Genes Dev. 10, 1455–1466.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., Nishizuka, Y., 1980. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinsotiol turnover. J. Biol. Chem. 2273–2276.

Kitatani, K., Idkowiak-Baldys, J., Hannun, Y.A., 2007. Mechanism of inhibition of sequestration of protein kinase C-α/βII by ceramide: roles of ceramide-activated protein phosphatases and phosphorylation/dephosphorylation of protein kinase C-α/βII on threonine 638/641. J. Biol. Chem. 282, 20647–20656.

Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., Rapp, U.R., 1993. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364, 249–252.

Kulukian, A., Fuchs, E., 2013. Spindle orientation and epidermal morphogenesis. Phil. Trans. R. Soc. B 368, 20130016.

Larsson, C., 2006. Protein kinase C and the regulation of the actin cytoskeleton. Cell Signal 18, 276–284.

Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P., Parker, P.J., 1998. Protein kinase C isotypes controlled by phosphoinosited 3-kinase through the protein kinase PDK1. Science 281, 2042–2045.

Lee, W., Haslinger, A., Karin, M., Tjian, R., 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature 325, 368–372.

Leonard, T.A., Rozycki, B., Saidi, L.F., Hummer, G., Hurdley, J.H., 2011. Crystal structure and allosteric activation of protein kinase C βII. Cell 144, 55–66.

Li, J.-J., Xie, D., 2014. RACK1, a versatile hub in cancer. Oncogene 1–9.

Ling, H., Sayer, J.M., Plosky, B.S., et al., 2004. Crystal structure off a benzo[a]pyrene diol epoxide adduct in a ternary complex with a DNA polymerase. Proc. Natl. Acad. Sci. USA. 101, 2265–2269.

Lopez-Bergami, P., Habelhah, H., Bhomik, A., Zhang, W., Wang, L.H., Ronai, Z., 2005. RACK1 mediates activation of JNK by protein kinase C. Mol. Cell 19, 309–320.

Lopez-Bergami, P., Huang, C., Goydos, J.S., et al., 2007. Rewired ERK-JNK signaling pathways in melanoma. Cancer Cell 11, 447–460.

Lu, M.S., Johnston, C.A., 2013. Molecular pathways regulating mitotic spindle orientation in animal cells. Development 140, 1843–1856.

Lu, H., Clauser, K.R., Tam, W.L., Fröse, J., Ye, X., Eaton, E.N., Reinhard, F., Donnenberg, V.S., Bhargava, R., Carr, S.A., Weinberg, R.A., 2014. Nat. Cell Biol. 16, 1105–1117.

Mani, S.A., Gu, W., Liao, M.-J., Eaton, E.N., Yyyanan, A., Zhou, A.Y., Brooks, M.M., Reinhard, F., Zhang, C.C., Shipitsin, M., Campbell, L.L., Polyak, K., Birsken, C., Yange, J., Weinberg, R.A., 2008. The epithelial-mysenchymal transition generates cells with properties of stem cells. Cell 133, 704–715.

Martin-Belmonte, F., Perez-Moreno, M., 2012. Epithelial cell polarity, stem cells and cancer. Nat. Rev. Cancer 12, 23–38.

Martin-Belmonte, F., Gassama, A., Datta, A., et al., 2007. PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. Cell 128, 383–397.

Mochly-Rosen, D., Das, K., Grimes, K.V., 2012. Protein kinase C, an elusive therapeutic target. Nat. Rev. Drug Discov. 11, 937–957.

Morais-de-Sa, E., Mirouse, V., St Johnston, D., 2010. aPKC phopshorylation of Bazooka defines the apical/lateral border in *Drosophila* epithelial cells. Cell 141, 509–523.

Moscat, J., Diaz-Meco, M.T., Albert, A., Campuzano, S., 2006. Cell signaling and function organized by PB1 domain interactions. Mol. Cell 5, 631–640.

Moscat, J., Diaz-Meco, M.T., Wooten, M.W., 2007. Signal integration and diversification through the p62 scaffold protein. Trends Biochem. Sci. 32, 95–100.

Mukhopadhyay, A., Saddoughi, S.A., Song, P., Sultan, I., Ponnusamy, S., Senkal, C.E., Snook, C.F., Arnold, H.K., Sears, R.C., Hannun, Y.A., Ogretmen, B., 2009. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of phosphatase 2A activity and signaling. FASEB J. 23, 751–763.

Munro, E., Nance, J., Priess, J.R., 2004. Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early *C. elegans* embryo. Dev. Cell 7, 413–424.

Myllymaki, S.M., Teravainen, T.P., Manninen, A., 2011. Two distinct integrin-mediated mechanisms contribute to apical lumen formation in epithelial cells. PLoS One 6, e19453.

Nakaya, Y., Sheng, G., 2013. EMT in developmental morphogenesis. Cancer Lett. 341, 9–15.

Newton, A.C., 1995. Protein kinase C. Seeing two domains. Curr. Biol. 5, 973–976.

Ngok, S.P., Lin, W.-H., Anastasiadis, P.Z., 2014. Establishment of epithelial polarity – GEF who's minding the GAP? J. Cell Sci. 127, 3205–3215.

Nilsson, J., Sengupta, J., Frank, J., Nissen, P., 2004. Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep. 5, 1137–1141.

Nishikawa, K., Toker, A., Johannes, F.J., Songyang, Z., Cantley, L.C., 1997. Determination of the specific substrate sequence motifs of protein kinase C isozymes. J. Biol. Chem. 952–960.

Nishimura, T., Yamaguchi, T., Kato, K., et al., 2005. PAR-6-PAR-3 mediates Cdc42-induced Rac activation through the Rac GEFs STEF/Tiam1. Nat. Cell Biol. 7, 270–277.

Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., Ichijo, H., 1998. ASK1 is essential for JNK/SAPK activation by TRAF2. Mol. Cell 2, 389–395.

Nishizuka, Y., 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308, 693–698.

Nishizuka, Y., 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 7, 484–496.

Oceana, E., Meyer, T., 1998. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 95, 307–318.

Ohno, S., Kawasaki, J., Imajoh, S., et al., 1987. Tissue-specific expression of three distinct types of rabbit protein kinase C. Nature 325, 161–166.

Osmani, N., Vitale, N., Borg, J.P., Etienne-Manneville, S., 2006. Scrib controls Cdc localization and activity to promote cell polarization during astrocyte migration. Curr. Biol. 16, 2395–2405.

Otte, A.P., Moon, R.T., 1992. Protein kinase C isozymes have distinct roles in neural induction and competence in Xenopus. Cell 68, 1021–1029.

Otte, A.P., Kramer, I.M., Durston, A.J., 1991. Protein kinase C and regulation of the local competence of Xenopus ectoderm. Science 251, 570–573.

Palmer, R.H., Ridden, J., Parker, P.J., 1994. Identification of multiple, novel, protein kinase C-related gene products. FEBS Lett. 356, 5–8.

Park, D.H., Rose, L.S., 2008. Dynamic localization of LIN-5 and GPR-1/2 to cortical for generation domains during spindle positioning. Dev. Biol. 315, 42–54.

Parker, P.J., Coussens, L., Totty, N., et al., 1986. The complete primary structure of protein kinase C – the major phorbol ester receptor. Science 233, 853–859.

Peterson, F.C., Penkert, R.R., Volkman, B.F., Prehoda, K.E., 2004. Cdc42 regulates the Par-6 PDZ domain through an allosteric CRIB-PDZ transition. Mol. Cell 13, 665–676.

Preitner, N., Quan, J., Nowakowski, D.W., Hancock, M.L., Shi, J., Tcherkezian, J., Young-Pearse, T.L., Flanagan, J.G., 2014. APC is an RNA-binding protein, and its interactome provides a link to neural development and microtubule assembly. Cell 158, 368–382.

Prevostel, C., Martin, A., Alvaro, V., Jaffiol, C., Joubert, D., 1997. Protein kinase Ca and tumorigenesis of the endocrine gland. Horm. Res. 47, 140–144.

Prevostel, C., Alvaro, V., Vallentin, A., Martin, A., Jaken, S., Joubert, D., 1998. Selective loss of substrate recognition induced by the tumour-associated D294G point mutation in protein kinase Ca. Biochem. J. 334, 393–397.

Rachfall, N., Schmitt, K., Bandau, S., Smolinski, N., Ehrenreich, A., Valerius, O., Braus, G.H., 2013. RACK1/Asc1P, a ribosomal node in cellular signaling. Mol. Cell Proteomics 12, 87–105.

Ranganathan, S., Wang, Y., Kern, F.G., Qu, Z., Li, R., 2007. Activation loop phosphorylation-independent kinase activity of human protein kinase C zeta. Proteins 15, 709–719.

Rath, O., Park, S., Tang, H.H., Banfield, M.J., Brady, R.L., Lee, Y.C., Dignam, J.D., Sedivy, J.M., Kolch, W., Yeung, K.C., 2008. The RKIP (Raf-1 Kinase Inhibitor Protein) conserved pocket binds to the phosphorylated N-region of Raf-1 and inhibits the Raf-1-mediated activated phosphorylation of MEK. Cell Signal 20, 935–941.

Repine, J.E., White, J.G., Clawson, C.C., Holmes, B.M., 1974. The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leukocytes. J. Lab. Clin. Med. 83, 911–920.

Rey, T., Morrison, S.J., Clarke, M.F., Weissman, I.L., 2001. Stem cells, cancer, and cancer stem cells. Nature 414, 105–111.

Ringer, S., 1888. A Handbook of Therapeutics. HK Lewis, London.

Ron, D., Chen, C.H., Caldwell, J., Jamieson, L., Orr, E., Mochly-Rosen, D., 1994. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. Proc. Natl. Acad. Sci. USA. 91, 839–843.

Rosenstreich, D.L., Mizel, S.B., 1979. Signal requirements for T lymphocyte activation, I. Replacement of macrophage function with phorbol myristic acetate. J. Immunol. 123, 1749–1754.

Rosentahl, A., Rhee, L., Yadegari, R., Paro, R., Ullrich, A., Goeddel, D.V., 1987. Structure and nucleotide sequence of a *Drosophila melanogaster* protein kinase C gene. EMBO J. 6, 433–441.

Sando, J.J., Chertihin, O.I., 1996. Activation off protein kinase C by lysophosphatidic acid: dependence on composition of phospholipid vesicles. Biochem. J. 317, 583–588.

Sarrio, D., Rodriguez-Pinilla, S.M., Hardisson, D., Cano, A., Moreno-Bueno, G., Palacios, J., 2008. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res. 68, 989–997.

Sassone-Corsi, P., Ransone, L.J., Lamph, W.W., Verma, I.M., 1988. Direct interaction between fos and jun nuclear oncoproteins: role of the "leucine-zipper" domain. Nature 336, 692–695.

Sato, N., Sanjuan, I.M., Heke, M., Uchida, M., Naef, F., Brivanlou, A.H., 2003. Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev. Biol. 260, 404–413.

Schackleton, M., Quantana, E., Fearon, E.R., Morrison, S.J., 2009. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138, 822–929.

Schober, M., Schaefer, M., Knoblich, J.A., 1999. Bazooka recruits inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. Nature 402, 548–551.

Schonwasser, D.C., Marais, R.M., Marschall, C.J., Parker, P.J., 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel and atypical protein kinase C isotypes. Mol. Cell Biol. 18, 790–798.

Schwamborn, J.C., Puschel, A.W., 2004. The sequential activity of the GTPase Rap1B and Cdc42 determine neuronal polarity. Nat. Neurosci. 7, 923–929.

Sharkey, N.A., Leach, K.L., Blumberg, P.M., 1984. Competitive inhibition by diacylglycerol of specific phorbol ester binding. Proc. Natl. Acad. Sci. USA. 81, 607–610.

Sivak, A., van Duuren, B.L., 1967. Phenotypic expression of transformation: inducting in cell culture by a phorbol ester. Science 157, 1443–1444.

Smeal, T., Binetruy, B., Mercola, D.A., Birrer, M., Karin, M., 2000. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature 354, 494–496.

Smith, C.A., Lau, K.M., Rahmani, Z., Dho, S.E., Brothers, G., She, Y.M., Berry, D.M., Bonneil, E., Thibault, P., Schweisguth, F., Le Borgne, R., McGlade, C.J., 2007. APKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. EMBO J. 26, 468–480.

Song, H.Y., Regnier, C.H., Kirschning, C.J., Goeddel, D.V., Rothe, M., 1997. Tumor necrosis factor (TNF)-mediated kinase cascades; bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor associated factor 2. Proc. Natl. Acad. Sci. USA. 94, 9792–9796.

Sozerie, O., Vollmer, K., Liayanage, M., Frith, D., Kour, G., Mark, G.E., Stael, S., 1992. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene 7, 2259–2262.

Srivastava, J., Goris, J., Dilworth, S.M., Parker, P.J., 2002. Dephosphorylation of PKCd by protein phosphatase 2AC and its inhibition by nucleotides. FEBS Lett. 516, 265–269.

Staudinger, J., Lu, J., Olson, E.N., 1997. Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-alpha. J. Biol. Chem. 272, 32019–32124.

Steinberg, S.F., 2008. Structural basis of protein kinase C isoform function. Physiol. Rev. 88, 1341–1378.

Stratton, M.R., Campbell, P.J., Futreal, P.A., 2009. The cancer genome. Nature 458, 719–724.

Sugiarto, S., Persson, A.I., Munoz, E.G., Waldhuber, M., Lamagna, C., Andor, N., Hanecker, P., Ayers-Ringler, J., Phillips, J., Siu, J., Lim, D.A., Vandenberg, S., Stallcup, W., Berger, M.S., Bergers, G., Weiss, W.A., Petritsch, C., 2011. Asymmetry-defective oligodendrocyte progenitors are glioma precursors. Cancer Cell 20, 328–340.

Sun, Z., Arendt, C.W., Ellmeier, et al., 2000. PKCtheta is required for TCR-induced NF-kB activation in mature but not immature T lymphocytes. Nature 23, 402–407.

Tabuse, Y., 2002. Protein kinase C isotypes in *C. elegans*. J. Biochem. 132, 519–522.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.

Takai, Y., Kishimoto, A., Inoue, M., Nishizuka, Y., 1977. Studies on a cyclic nucleotide independent protein kinase and it proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. J. Biol. Chem. 252, 7603–7609.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nishhizuka, Y., 1979. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J. Biol. Chem. 254, 3692–3695.

Tam, W.L., Lu, H., Buikhuisen, J., Soh, B.S., Lim, E., Reinhardt, F., Wu, Z.J., Krall, J.A., Bierie, B., Guo, W., Chen, X., Liu, X.S., Brow, M., Lim, B., Weinberg, R.A., 2013. Protein kinase C α is a central signaling node and therapeutic target for breast cancer stem cells. Cancer Cell 24, 347–364.

Tan, W., Zhang, W., Strasner, A., Givennikov, S., Cheng, J.Q., Hoffman, R.M., Karin, M., 2011. Tumour-infiltrating regulatory T-cells stimulate mammary cancer metastasis through RANKL-RANK signalling. Nature 470, 548–553.

Tavel, L., Jaquillard, L., Karsisiotis, A.E., Saab, F., Jouvensal, L., Brans, A., Delmas, A.F., Schjoentgen, F., Cadene, M., Damblon, C., 2012. Ligand binding study of human PEBP1/RKIP: interaction with nucleotides and Raf-1 peptides evidenced by NMR and mass spectrometry. PLoS ONE 7, e36187.

Ten Klooster, J.P., Jaffer, Z.M., Chernoff, J., Hordijk, P.L., 2006. Targeting and activation of Rac1 are mediated by the exchange factor bPix. J. Cell Biol. 172, 759–769.

Tian, A.G., Deng, W.M., 2008. Lgl and its phosphorylation by aPKC regulate oocyte polarity formation in *Drosophila*. Development 135, 463–471.

Tognon, C.E., Kirk, H.E., Passmore, L.A., Whitehead, I.P., Der, C.J., Kay, R.J., 1998. Regulation of RasGRP via a phorbol ester-responsive C1 domain. Mol. Cell Biol. 18, 6995–7008.

Toker, A., Meyer, M., Reddy, K.K., et al., 1994. Activation of protein kinase C family members by the novel polyphosphoinositides Ptdlns-3,4-P2 and Ptdlns-3,4,5-P3. J. Biol. Chem. 269, 32358–32367.

Topman, M.K., 2006. Signaling roles of diacyglycerol kinases. J. Cell Biochem. 97, 474–484.

Treisman, R., 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. Cell 42, 889–902.

Treisman, R., 1986. Identification of a protein-binding site that mediates transcriptional response of the c-fos gene to serum factors. Cell 46, 567–574.

Tsai, F.-C., Seki, A., Yang, H.W., Hayer, A., Carrasco, S., Malmersjo, S., Meyer, T., 2014. A polarized Ca^{2+}, diacylglycerol and STIM1 signalling system regulates directed cell migration. Nat. Cell Biol. 16, 133–144.

van Baal, J., de Widt, J., Divecha, N., van Blitterswijk, W.J., 2005. Translocation of diacylglycerol kinase θ from cytosol to plasma membrane in response to activation of G protein-coupled receptors and protein kinase C. J. Biol. Chem. 280, 9870–9878.

van Duuren, B.L., Orris, L., Arroyo, E., 1963. Tumour enhancing activity of the active principle off Croton tiglium. Nature 2000, 1115–1116.

Verdaguer, N., Corbalan-Garcia, S., Ochoa, W.F., Fita, I., Gomez-Fernandez, J.C., 1999. Ca^{2+} bridges the C2 membrane-binding domain of protein kinase Ca directly to phosphatidylserine. EMBO J. 18, 6329–6338.

Vessey, J.P., Amadei, G., Burns, S.E., Kiebler, M.A., Kaplan, D.R., Miller, F.D., 2012. An asymmetric localized staufen2-dependent RNA complex regulates maintenance of mammalian neural stem cells. Cell Stem Cell 11, 517–528.

Vial, E., Sahai, E., Marshall, C.J., 2003. ERK-MAPK signaling coordinately regulates activity off Rac1 and RhoA for tumor cell motility. Cancer Cell 4, 67–79.

Visvader, J.E., 2011. Cells of origin in cancer. Nature 469, 314–322.

Vogt, P.K., 2001. Jun, the oncoprotein. Oncogene 20, 2365–2377.

Walther, R.F., Pichaud, F., 2010. Crumbs/DaPKC-dependent apical exclusion of Bazooka promotes photoreceptor polarity remodeling. Curr. Biol. 20, 1065–1074.

Wang, C., Shang, Y., Zhang, M., 2012. Substrate recognition mechanism of atypical protein Cs revealed by the structure of PKCi in complex with a substrate peptide from Par-3. Structure 20, 791–801.

Wedlicht-Soldner, R., Altschuler, S., Wu, L., Li, R., 2003. Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. Science 299, 1231–1235.

Williams, S.E., Beronja, S., Pasolli, H.A., Fuchs, E., 2011. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. Nature 470, 353–358.

Williams, S.E., Ratcliff, L.A., Postiglione, P., Knoblich, J.A., Fuchs, E., 2014. Par3-Minsc and Gai3 cooperate to promote oriented epidermal cell divisions through LGN. Nat. Cell Biol. 16, 758–769.

Wilson, M.I., Gill, D.J., Perisic, O., Quinn, M.T., Williams, R.L., 2003. PB1 domain-mediated heterodimerization in NADPH oxidase and signaling complexes of atypical protein kinase C with Par6 and p62. Mol. Cell 12, 39–50.

Wodarz, A., Ramrath, A., Kuchinke, U., Knust, E., 1999. Bazooka provides an apical cue for inscuteable localization in *Drosophila* neuroblasts. Nature 402, 544–547.

Yazawa, S., Kamakura, S., Iwakiri, Y., Hayase, J., Sumimoto, H., 2011. Structural basis for interaction between the conserved cell polarity proteins inscuteable and Leu-gly-Asn repeat-enriched protein (LGN). Proc. Natl. Acad. Sci. USA. 108, 19210–19215.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W., 1999. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature 401, 173–177.

Zenz, R., Eferl, R., Scheinecker, C., Redlich, K., Smolen, J., Schontaler, H.B., Kenner, L., Tschachler, E., Wagner, E.F., 2008. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. Arthritis Res. Ther. 10, 201.

Zhang, G., Kazanietz, M.G., Blumberg, M.M., Hurley, J.H., 1995. Crystal structure of the cys2 activator-binding domain of protein kinase Cd in complex with phorbol ester. Cell 81, 917–924.

Zhang, H., Zhang, R., Luo, Y., D'Alessio, A., Pober, J.S., Min, W., 2004. AIP1/DAB2IP, a novel member of the Ras-GAP family, transduces TRAF2-induced ASK1-JNK activation. J. Biol. Chem. 279, 44955–44965.

Zhang, H., Zhang, H., Lin, Y., Li, J., Pober, J.S., Min, W., 2007. RIP1-mediated AIP1 phosphorylation at a 14-3-3-binding site is critical for tumor necrosis factor-induced ASK1-JNK/p38 activation. J. Biol. Chem. 282, 14788–14796.

Zhang, Y., Wang, W., Chen, J., Zhang, K., Gao, F., Gao, B., Zhang, S., Dong, M., Besenbacher, F., Gong, W., Zhang, M., Sun, F., Feng, W., 2013. Structural insights into the intrinsic salf-assembly of Par-3 N-terminal domain. Structure 21, 997–1006.

Zhu, J., Wen, W., Zheng, Z., Shang, Y., Wei, Z., Xiao, Z., Pan, Z., Du, Q., Wang, W., Zhang, M., 2011. LGN/mInsc and LGN/NuMa complex structures suggest distinct functions in asymmetric cell division for Par3/Insc/LGN and Gai/LGN/NuMa pathways. Mol. Cell 43, 418–431.

Zihni, C., Munro, P.M.G., Elbediwy, A., Keep, N.H., Terry, S.J., Harris, J., Balda, M.S., Matter, K., 2014. Dbl3 drives Cdc42 signaling at the apical margin to regulate junction position and apical differentiation. J. Cell Biol. 204, 111–127.

CHAPTER

10

Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases

INTRODUCTION

Of the 90 genes that code for tyrosine protein kinases in the human genome, 58 are of the receptor type (rPTK), distributed into 20 subfamilies. The remaining 32 are of the non-receptor type (nrPTK) or also known as cytoplasmic tyrosine kinases. Mouse homologs have been identified for nearly all of the human tyrosine kinases (Robinson et al., 2000). Tyrosine protein kinases have long been considered a hallmark of intercellular communication, unique to multicellular animals, but this is no longer valid. The tyrosine kinase may be less abundant in bacteria but, over the last 10 years, biochemical, phosphoproteomic, and genomic analyses have revealed that bacteria contain phosphotyrosines and they contain at least three eukaryotic-like tyrosine kinases. A first clear example came from studies on *Acinobacter johnsonii*, which contains an 81 kDa protein, named Ptk, that autophosphorylates on tyrosine (Grangeasse et al., 1997). *Escherichia coli* also carries a protein tyrosine kinase, named Wzc (Vincent et al., 1999). Protists, unicellular eukaryotes which are considered to be the closest known relatives of multicellular animals (metazoans), also express tyrosine kinases. One of its members, the choanoflagellate *Monosiga brevicollis*, has a remarkable count of 128 and it possesses a tyrosine-kinase signaling network more elaborate than found in any known metazoan (Manning et al., 2008; Suga et al., 2012). It is suggested that receptor tyrosine kinases that had been used for receiving environmental cues in unicellular organisms were subsequently used as a communication tool between cells at the onset of metazoan multicellularity.

Here we consider the receptor protein tyrosine kinases that exist as integral domains of transmembrane receptors in mammals. In Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases," we discuss the non-receptor protein tyrosine kinases that are present in the cytosol or are attached to the plasma membrane and that can be recruited to receptors.

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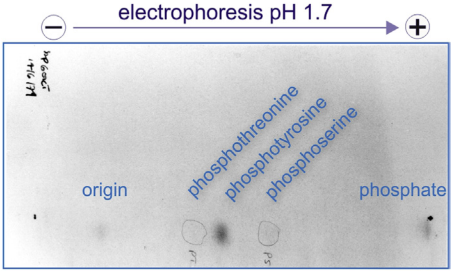


FIGURE 10-1 Separation of phosphotyrosine from phosphoserine and phosphothreonine by paper electrophoresis. *Courtesy Tony Hunter.*

SPOTTING PHOSPHOTYROSINE

Here is another turning point in science that owed as much to chance as to the application of a well-prepared mind. This involved Tony Hunter and the discovery of tyrosine phosphorylation of proteins associated with malignant transformation. He was interested in identifying the transforming antigens of the tumor-causing polyoma virus, of which the main component is the so-called middle T-antigen. A report that the src-gene product (v-Src) was associated with protein kinase activity begged the question whether other tumor virus gene products might also possess phosphorylating activities and that this might underlie cell transformation (Collett and Erkison, 1978; Bazley and Gullick, 2005). It became apparent that infection with the polyoma virus induces extensive phosphorylation of cellular protein, but that the transforming protein, middle T-antigen itself, also becomes phosphorylated. After proteolytic digestion of ^{32}P-labeled protein into its component amino acids, the labeled residues were separated by electrophoresis. Unexpectedly, all of the label was confined to a new spot, now known to be due to phosphotyrosine (Figure 10-1) (Eckhart et al., 1979).

In a sense, this discovery was accidental. It was common practice when carrying out electrophoretic procedures to reuse the buffers on subsequent occasions. Eventually, the pH must alter the anodic buffer becoming more acidic and the cathodic more alkaline. Had the pH 1.9 electrophoresis buffer been freshly prepared, the separation of phosphotyrosine would not have occurred. In the event, with reuse, it had become more acidic (pH 1.7). As a consequence the phosphotyrosine migrated more slowly and was separated from the phosphothreonine.

v-Src AND OTHER PROTEIN TYROSINE KINASES

With electrophoresis now carried out intentionally at pH 1.7, various other labeled protein digests were tested and it was found that v-Src can phosphorylate tyrosine residues on a range of quite unrelated proteins.

This identified v-Src as a protein tyrosine kinase and phosphorylation on tyrosine residues to be an authentic physiological process. Confirmation came with the finding that while labeling in non-transformed cells occurs almost exclusively upon serines and threonines, in cells transformed by Rous sarcoma virus, phosphorylated threonine, serine, and tyrosine residues are present in almost equal amounts.

It was also found that the transforming protein of the Abelson murine leukemia virus (v-Abl) becomes labeled on a tyrosine residue when incubated in vitro with ^{32}P-ATP (Witte et al., 1980). The target in this case is the tyrosine kinase itself, an example of auto-phosphorylation. Importantly, a second look at the phosphorylation status of the epidermal growth factor receptor (EGFR) showed that the labeling due to ligand binding occurs on tyrosine, not threonine residues as previously reported (Ushiro and Cohen, 1980). The link between tyrosine phosphorylation and cell proliferation/transformation had been established.

DETECTING PHOSPHOTYROSINE

Since the phosphate–tyrosine bond is comparatively resistant to alkali, the detection of tyrosine phosphorylation was simplified by treating ^{32}P-labeled cellular extracts with 1M NaOH. More recently, antibodies have been developed that specifically recognize individual phosphotyrosine epitopes, and this of course makes detection a very clean affair. The creation of antibodies of high specificity is now a business in its own right.

Tyrosine phosphorylation is not limited to the actions of the transforming viruses or epidermal growth factor. It regulates many other important signaling processes including:

- Cell–cell and cell–matrix interactions through integrin receptors and focal adhesion sites (Giancotti, 1997; Miettine et al., 1995) (Chapter 11, “Signal Transduction to and from Adhesion Molecules”).
- Stimulation of the respiratory burst in phagocytic cells, such as neutrophils and macrophages (Naccache et al., 1990).
- Activation of B lymphocytes by antigen binding to the B cell receptor (Burg et al., 1994).
- Activation of T lymphocytes by antigen-presenting cells through the T cell receptor complex (Chapter 15, “Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases”) (Cantrell, 1996).
- Interleukin-2-mediated proliferation of lymphocytes (Williamson et al., 1994; Kirken et al., 1993).
- Activation of mast cells and basophils through the high-affinity receptor for IgE (Li et al., 1992).
- Many developmental processes.

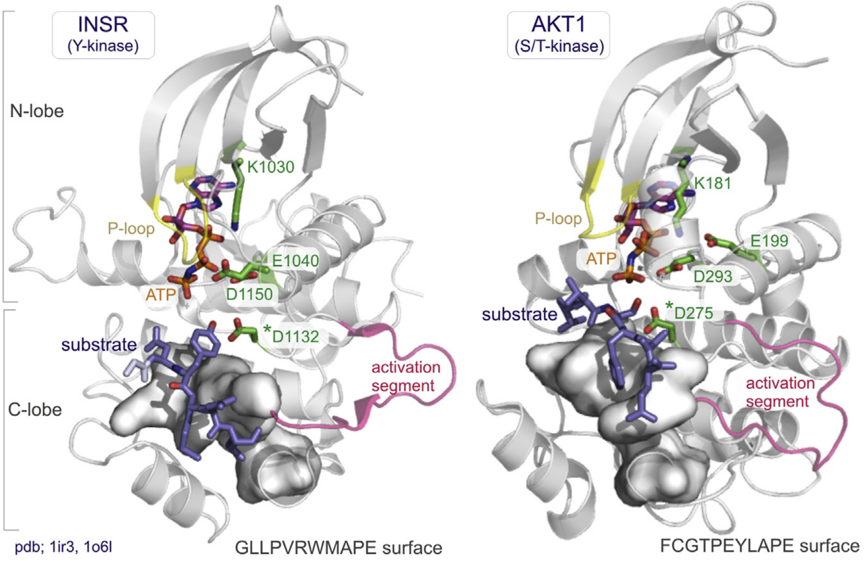


FIGURE 10-2 Comparing the structure of serine/threonine with tyrosine protein kinase. Both types of protein kinases manifest similar kinase folds with identical highly conserved residues that play a role in the positioning of ATP (K, E, and D) and catalysis (*D1132 on the left, or *D275 on the right). The substrate-binding segment (relevant sequence shown underneath the kinases) is represented in surface mode in order to highlight the more outward position of the tyrosine-containing peptide (on the left). If tyrosine would be replaced by a serine or threonine, its OH group would not reach the catalytic residue within a distance of 0.3 nm. The serine-containing peptide shown on the right penetrates further into the catalytic cleft.

Here, we focus on the signal transduction pathway initiated by the binding of EGF, and other ligands of the EGF family, to receptors on fibroblasts or epithelial cells. A number of the principles also apply for other tyrosine kinase-containing receptors.

Tyrosine protein kinases have a near identical kinase fold as the serine/threonine kinases dealt with previously, but they differ in their substrate-binding site. In general, the substrate penetrates much less into the catalytic cleft and thus requires a long amino acid, which is tyrosine, to reach both the catalytic aspartate and the γ-phosphate of ATP (Figure 10-2). Binding of serine or threonine within the same sequence motif would simply not work because the OH-group remains out of reach. This is not an exclusive reason for substrate selectivity; other aspects certainly play a role. For a detailed analysis of the protein kinase fold and its essential (conserved) residues, return to Chapter 2, "An Introduction to Signal Transduction," Section "Protein kinases structure and function" (Figure 2-35).

The 58 receptor types are distributed into 19 subfamilies (Figure 10-3), and of these we focus on the prototypic EGFR (member of the ERBB subfamily).

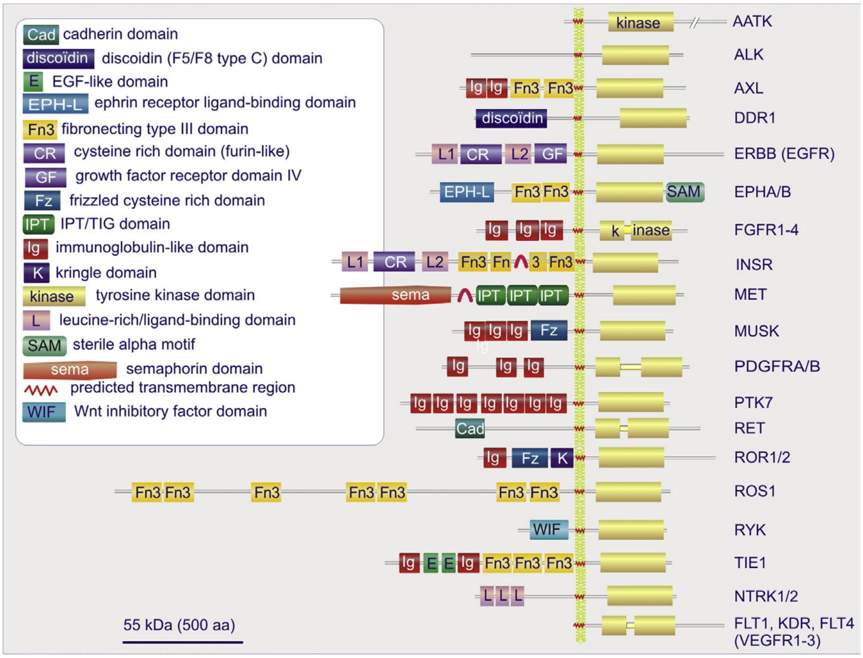

Cad cadherin domain
discoïdin discoidin (F5/F8 type C) domain
E EGF-like domain
EPH-L ephrin receptor ligand-binding domain
Fn3 fibronecting type III domain
CR cysteine rich domain (furin-like)
GF growth factor receptor domain IV
Fz frizzled cysteine rich domain
IPT IPT/TIG domain
Ig immunoglobulin-like domain
K kringle domain
kinase tyrosine kinase domain
L leucine-rich/ligand-binding domain
SAM sterile alpha motif
sema semaphorin domain
predicted transmembrane region
WIF Wnt inhibitory factor domain
55 kDa (500 aa)
kinase
AATK
ALK
Ig Ig Fn3 Fn3
AXL
discoïdin
DDR1
L1 CR L2 GF
ERBB (EGFR)
EPH-L Fn3 Fn3 SAM
EPHA/B
Ig Ig Ig k inase
FGFR1-4
L1 CR L2 Fn3 Fn 3 Fn3
INSR
sema IPT IPT IPT
MET
Ig Ig Ig Fz
MUSK
Ig Ig Ig
PDGFRA/B
Ig Ig Ig Ig Ig Ig Ig
PTK7
Cad
RET
Ig Fz K
ROR1/2
Fn3 Fn3 Fn3 Fn3 Fn3 Fn3 Fn3
ROS1
WIF
RYK
Ig E E Ig Fn3 Fn3 Fn3
TIE1
L L L
NTRK1/2
FLT1, KDR, FLT4 (VEGFR1-3)

FIGURE 10-3 Classification of tyrosine kinase-containing receptors. All these receptors possess a single membrane-spanning segment and all of them carry a tyrosine kinase catalytic domain, in some cases interrupted by an insert. The extracellular domains vary as indicated. The domain architectures are obtained from Pfam. *Adapted from* *Robison et al. (2000)*.

Web resource

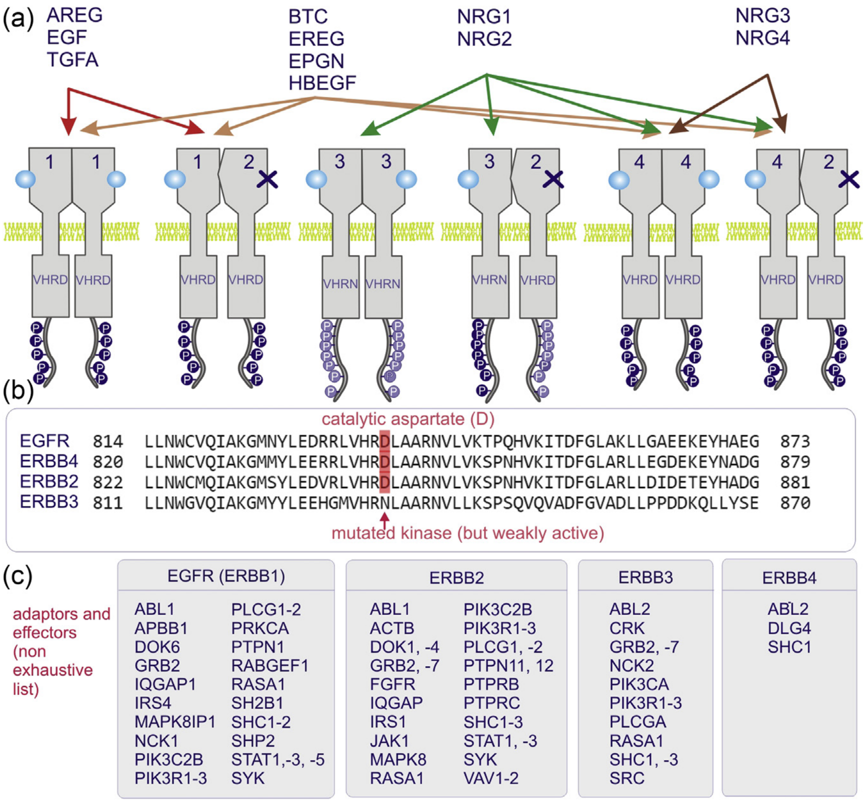
The PFAM database (Protein Family, Multiple Sequence Alignment and hidden Markov models), run by EMBL-EBI, offers multiple ways to analyze protein sequences. The graphic display of protein domains is one way of getting insight into the possible functional qualities of a protein. You can access directly through http://pfam.xfam.org and search with UniProt entry codes or enter via UniProt and go to paragraph "Family and Domains" (Finn et al., 2014).

FOCUS ON THE ERBB RECEPTOR FAMILY, THEIR LIGANDS, AND THEIR DIMER PARTNERS

The first receptor tyrosine kinase discovered was the EGF receptor (Carpenter et al., 1979). It was also the first receptor that provided evidence for a relationship between activating mutations (oncogenes) and cancer. The EGF receptor is a member of the ERBB family of proteins (EGFR, ERBB2, -3, and -4) which act as dimers in different combinations and with different ligands (Figure 10-4). Of these, ERBB2 lacks the capacity to interact with a ligand (Cho et al., 2003) because of a closed binding site, and ERBB3 has impaired kinase activity because its catalytic aspartate is replaced by an arginine (within the sequence motif HRD) (Guy et al., 1994). These so-called nonautonomous receptors do, however, contribute to growth factor signaling because they can form heterodimeric complexes in which ERBB2 is the preferred dimerization partner. Association of ERBB2 with, for instance, the ligand-bound EGFR increases both the intensity and duration of the EGF signal, by increasing the ratio of active kinase to EGF and by inhibiting receptor uptake in clathrin-coated endocytic vesicles (Hendriks et al., 2005). ERBB2 can be said to act as an amplifier of the ERBB signaling network (Citri and Yarden, 2006) and this may explain why ERBB2-gene anomalies are involved in numerous cancers such as hereditary diffuse gastric cancer, glioma, ovarian, lung, gastric, and breast cancer (search OMIM for entry codes 137215, 137800, 167000, 211980, 613659, and 114480). As a consequence, both the kinase and extracellular segment of ERBB2 are much sought after targets in the development of inhibitors for cancer therapy. This frenzy is witnessed by the 21 PDB entries (you can find them in the paragraph "structure" of the UniProtKB database). In the context of cancer, members of the ERBB receptors are often referred to as Her1, Neu/Her2, Her3, and Her4 (which are abbreviations for human epidermal growth factor receptor and neuro/glioblastoma transforming gene) (Schechter et al., 1984).

The ERBB2/ERBB3 partnership has been dubbed "the deaf and the dumb," but this does not prevent them from being a potent signaling couple (Citri et al., 2003). Surprisingly, despite lacking the catalytic aspartate (see Figure 10-4(b)), low levels of receptor phosphorylation has been detected in ERBB3 homodimers and in the ERBB2 receptor of an ERBB2/ERBB3 heterodimer. This means that the kinase is active toward both ERRB2 and ERBB3 but no other substrates have yet been revealed (Shi et al., 2010; Steinkamp et al., 2014).

ERBB is so named after the viral oncogene product, erb-B, of the erythroblastoma virus to which they are related. The official gene name for ERBB1 is EGFR. The ErbB family is an evolutionary elaboration of the Let-23 ancestral gene that is present in *Caenorhabditis elegans* and its equivalent DER (also known as torpedo) in *Drosophila melanogaster.* Gene duplication gave rise to two-member EGFR families in early vertebrate species such as fishes. Further gene duplication generated the four members of the mammalian ERBB family. An increasing diversity of extracellular ligands developed in parallel (Stein and Staros, 2000).



```
EGFR  814  LLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGAEEKEYHAEG  873
ERBB4 820  LLNWCVQIAKGMMYLEERRLVHRDLAARNVLVKSPNHVKITDFGLARLLEGDEKEYNADG  879
ERBB2 822  LLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADG  881
ERBB3 811  LLNWGVQIAKGMYYLEEHGMVHRNLAARNVLLKSPSQVQVADFGVADLLPPDDKQLLYSE  870
```

EGFR (ERBB1)		ERBB2		ERBB3	ERBB4
ABL1	PLCG1-2	ABL1	PIK3C2B	ABL2	ABL2
APBB1	PRKCA	ACTB	PIK3R1-3	CRK	DLG4
DOK6	PTPN1	DOK1, -4	PLCG1, -2	GRB2, -7	SHC1
GRB2	RABGEF1	GRB2, -7	PTPN11, 12	NCK2	
IQGAP1	RASA1	FGFR	PTPRB	PIK3CA	
IRS4	SH2B1	IQGAP	PTPRC	PIK3R1-3	
MAPK8IP1	SHC1-2	IRS1	SHC1-3	PLCGA	
NCK1	SHP2	JAK1	STAT1, -3	RASA1	
PIK3C2B	STAT1,-3, -5	MAPK8	SYK	SHC1, -3	
PIK3R1-3	SYK	RASA1	VAV1-2	SRC	

FIGURE 10-4 ERBB receptor family, their ligands, and some of their adaptors and effectors. (a) Each receptor binds different ligands with the exception of ERBB2 which remains in an unliganded state. ERBB2 homodimers may occur but are instable. (b) Sequence alignment of the kinase domain shows that the essential catalytic aspartate (D) is replaced by an arginine (N) in ERBB3. Nevertheless the receptor is not entirely kinase dead and can phosphorylate its counterpart (phosphorylation in *trans* of either ERBB2 or ERBB3). (c) Listing of some adaptors and effectors that associate with the phosphotyrosines of the intracellular segment of ERBB members. Information obtained from UniProtKB, paragraph "interactions," using the entry codes P00533, P04626, P21860, Q15303.

Three groups of ligands have been identified that contain one or more copies of a conserved EGF domain (30–40 residues). All of these bind to two or more of the receptors. Many of the ligands, including EGF, are expressed as large transmembrane proteins that can signal in a juxtacrine (cell–cell interaction) or paracrine mode, following proteolytic cleavage of their extracellular domain. In the case of human EGF, only 55 residues constitute the growth factor out of more than 1200 present in its membrane-tethered precursor form.

The various ligands have redundant functions, well exemplified by deletion of TGF-α, EGF, and amphiregulin (AREG). Single knockouts have little effect on developmental processes; indeed, it takes a triple deletion to manifest serious effects in mice (Luetteke et al., 1993, 1999). By contrast, inactivation of the EGFR results in mid-gestational death (Threadgill et al., 1995).

CROSS-LINKING OF RECEPTORS CAUSES ACTIVATION

Tyrosine kinase-containing receptors come in several different forms but they are unified by the presence of a single membrane-spanning domain and an intracellular kinase catalytic domain. The extracellular chains vary considerably as indicated in Figure 10-3. A general feature of these receptors is that ligand binding causes dimerization, first discovered for the EGF receptor (Yarden and Schlessinger, 1987; Sternberg and Gullick, 1990). In addition to activation by the natural peptide ligands, some (but not all) of the functions of the EGF receptor can be elicited by crosslinking with antibodies (Defize et al., 1986; Spaargaren et al., 1991). Within the family of the receptor tyrosine kinases, ligand-mediated cross-linking is achieved in a number of ways. Platelet-derived growth factor (PDGF) is itself a disulfide-linked dimeric ligand which cross-links two receptors upon binding. FGF uses a heparin oligosaccharide to bring two receptors together. In the case of the insulin receptors, they are already dimerized through a disulfide bridge but nevertheless require insulin in order to signal into the cell (Ottensmeyer et al., 2000; Yip and Ottensmeyer, 2003). EGF is different, its binding causes the receptor to unfold, exposing a dimerization motif that allows the occupied monomers to recognize each other (or to recognize unliganded unfolded ERBB2) (Yamanaka et al., 2002; Gerrett et al., 2002) (Figure 10-5). Since truncated EGF receptors that lack the extracellular ligand-binding domain are constitutively active (Thiel and Carpenter, 2007), it follows that the unoccupied extracellular domain acts to prevent kinase activation. Indeed, dimerization of the receptor brings about a change in the relative position of the transmembrane domains that, in turn, promotes dimerization and activation of the intracellular protein kinase segment (Endres et al., 2013).

Normally, the kinase domain of the EGFR is inactive because both the activation segment and the αC- helix are wrongly positioned. Moreover, the kinase is tethered to the plasma membrane through both its N-terminal juxtamembrane sequence, which three leucines (motif LLRRL), and through interactions of surface lysines and arginines (positively charged) with negatively charged phospholipids (Figure 10-6) (Arkhipov et al., 2013). Membrane-tethered kinases cannot form dimers. Receptor crosslinking disrupts the membrane binding and allows the two kinases to align asymmetrically, with the C-lobe (activator kinase) binding to the N-lobe

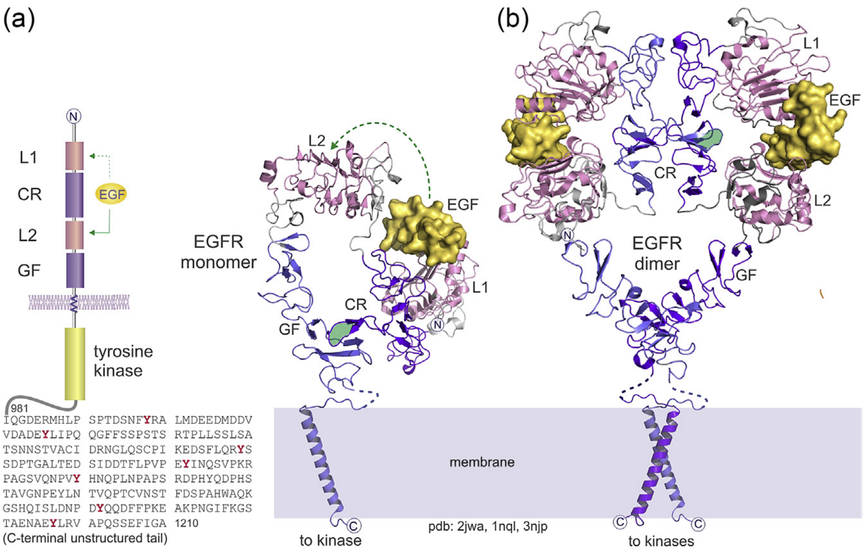

(a)
N
L1
CR
EGF
L2
GF
tyrosine kinase
981
IQGDERMHLP SPTDSNFYRA LMDEEDMDDV
VDADEYLIPQ QGFFSSPSTS RTPLLSSLSA
TSNNSTVACI DRNGLQSCPI KEDSFLQRYS
SDPTGALTED SIDDTFLPVP EYINQSVPKR
PAGSVQNPVY HNQPLNPAPS RDPHYQDPHS
TAVGNPEYLN TVQPTCVNST FDSPAHWAQK
GSHQISLDNP DYQQDFFPKE AKPNGIFKGS
TAENAEYLRV APQSSEFIGA 1210
(C-terminal unstructured tail)
L2
EGF
EGFR monomer
L1
CR
GF
N
C
to kinase
membrane
pdb: 2jwa, 1nql, 3njp
(b)
L1
EGF
CR
L2
N
EGFR dimer
GF
C
C
to kinases

FIGURE 10-5 Dimerization of the EGF-receptor extracellular segment. (a) The EGF receptor is composed of four extracellular domains, L1, CR, L2, and GH. L1 and L2 carry leucine-rich repeats that function in ligand binding, CR1 is a furin-like cysteine-rich domain and GH is a growth factor receptor domain IV. The intracellular segment contains a tyrosine kinase domain and a long unstructured C-terminal sequence that harbors numerous phosphorylation sites. The C-terminal tail is the substrate of a neighboring receptor (phosphorylation in *trans*). Note that in the receptor structure on the left, the extracellular segment folds onto itself; the dimerization arm of the CR domain (indicated by a green surface) docks onto the GF domain. The receptor shown is bound to EGF but only to the L1 domain. (b) Binding of EGF to both L1 and L2 causes unfolding and dimerization of the extracellular segment. The dimerization arm of CR now binds to a docking site in the CR domain of the partner (and vice versa). As a consequence, the two transmembrane domains associate with their N-terminal sequences and this, in turn, brings about a change in the position of the kinase domains leading to their activation (not shown, see Figure 10-6).

(receiver). This causes an outward movement of the activation segment and inward movement of the αC-helix of the receiver kinase (Figure 10-6). The other, activator kinase, remains catalytically incompetent. The EGFR is rendered competent through an allosteric interaction between kinase domains (in other words, an activating "shape change" is caused by protein–protein interaction) (Jura et al., 2009; Brewer et al., 2009). The interaction between the two EGFR-kinase domains is reinforced by the antiparallel association of the helices carrying the –LRRLL motif and by the juxtamembrane region of the receiver kinase which latches onto the activator (juxtamembrane latch). The mode of activation is unique among the tyrosine protein kinases, which otherwise often involves phosphorylation of the activation segment (further discussed in Chapter 15, "Activating the Adaptive Immune System: Role off Non-receptor Tyrosine Kinases" and Chapter 16,

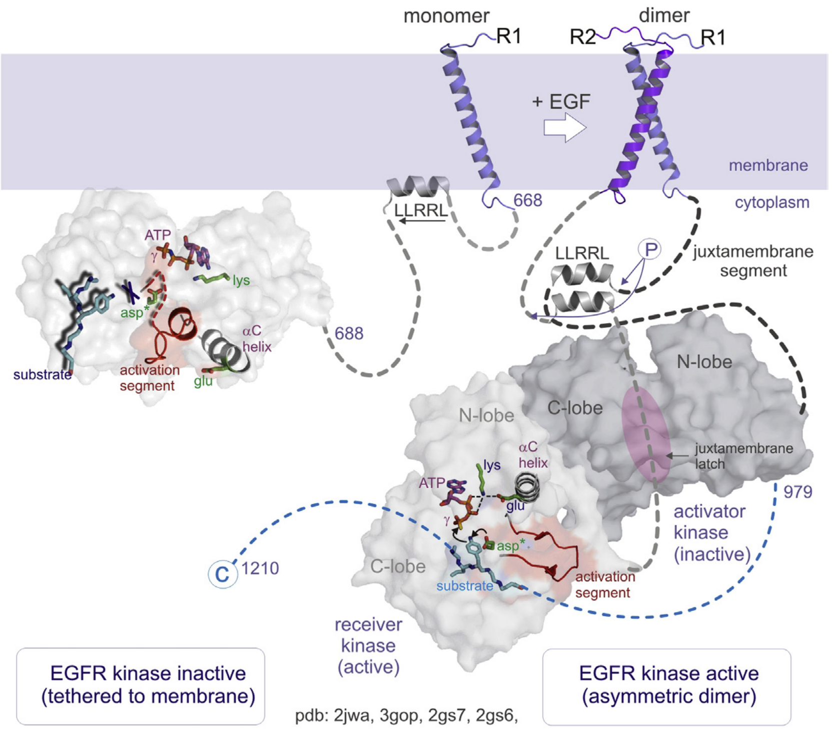


FIGURE 10-6 Allosteric regulation of the EGFR kinase domains through the formation of an asymmetric kinase dimer. In the inactive state (monomer) the kinase domain is tethered to the membrane through an interaction of the LRRLL motif in the juxtamembrane sequence and through binding of lysine residues of the kinase domain to negatively charged lipids. Ligand-mediated dimerization of the extracellular segment leads to dimerization of the transmembrane helix and this disrupts membrane attachment of the kinase domain. Two protein kinase domains now associate head-to-tail (asymmetric dimer) bringing about a conformational change of one kinase only, named the "receiver kinase." As a consequence, the multiple residues on the adjacent C-terminal tail are phosphorylated. As the kinase domain may change roles, where the activator becomes the receiver kinase, with time both tails will be fully phosphorylated. The phosphate symbol indicates a phosphorylation site (threonine-678) in the juxtamembrane region which inactivates the receptor (part of a negative feedback mechanism).The residues of the human EGFR are numbered according to the sequence information in UniProtKB (with inclusion of the signal peptides, 1–24).

"Signaling through the Insulin Receptor"). However, the mechanism is similar to the one observed with cyclin-dependent protein kinases (CDKs), which are serine/threonine protein kinases. Here allosteric regulation is brought about by a regulatory subunit, named cyclin, that binds the kinase domain.

It is likely that the receiver and activator positions can be interchanged, so that the kinases alternate their activity. Indeed EM studies of EGF-treated receptors have revealed that the dimeric extracellular segment is associated with intracellular segments of different conformations, ranging from asymmetric dimers (active), symmetric dimers (inactive) to nonassociated kinase domains (Mi et al., 2011). Importantly, as a consequence of asymmetric dimerization, the C-terminal tails of the two kinases are *trans*-phosphorylated (one kinase phosphorylates the tail of the other) on multiple tyrosine residues. The phosphorylated dimer then constitutes the active receptor. For more information about protein kinase activation, return to Chapter 2, “An Introduction to Signal Transduction,” Section “Protein kinase activation mechanisms” (Figures 2-43 and 2-44).

While certain proteins serve as substrates of the activated EGF receptors, what really matters is the recruitment of signaling partners through the phosphotyrosine residues in the C terminal tail. These phosphotyrosines, within a specific context of five amino acids, bind proteins bearing SH2 or PTB domains (Liu et al., 2010). The recruited proteins can be effectors or adaptors, which serve to bind effectors indirectly (Anderson et al., 1990; Jones et al., 2006) (see Figure 10-4 for a list of such effectors and adaptors, and see Figure 10-8 for more detail about the interaction).

Growth factor receptor dimers can further aggregate into oligomers of several hundreds or even thousands of units. This phenomenon, already recorded at the time when receptor dimerization first came to light (Yarden and Schlessinger, 1987), has now been visualized by the use of fluorescent protein tagging (Carter and Sorkin, 2006). Different types of receptors can be recruited, so that PDGF receptors have been found associated with EGF receptors (Saito et al., 2001) and these aggregates give rise to multiprotein signaling platforms that may contain numerous effectors.

ONCOGENENIC MUTATIONS

Oncogenic EGFR mutations have been detected in cancer. Examples are deletion mutations of the entire extracellular segment, deletion mutation of amino acids 746–750 or point mutations such as L858R in the activation segment (>50 increase in kinase activity) or G917S/S/A in the P-loop (same overall structure but higher affinity for ATP, >10 activity) (Yun et al., 2007). Below we explain why replacing leucine-858 with arginine increases kinase activity.

In the wild-type receptor two leucines which are part of the activation segment, at positions 858 and 861, wedge into the N-terminal lobe and push the αC-helix outward. As a consequence, the essential glutamate (E762) cannot contribute to the coordination of ATP. When the C-lobe of the activator kinase bumps onto the N-lobe of the receiver, the leucine wedge is pushed outward, allowing the αC-helix to move inward and the N-lobe to shift downward (closed conformation). This turns the kinase catalytically competent. Replacing a nonpolar leucine-858 with a positively charged arginine (R) brings about the same effect. Note the resemblance of the two structures between Figure 10-7(b) and (c). The above-mentioned

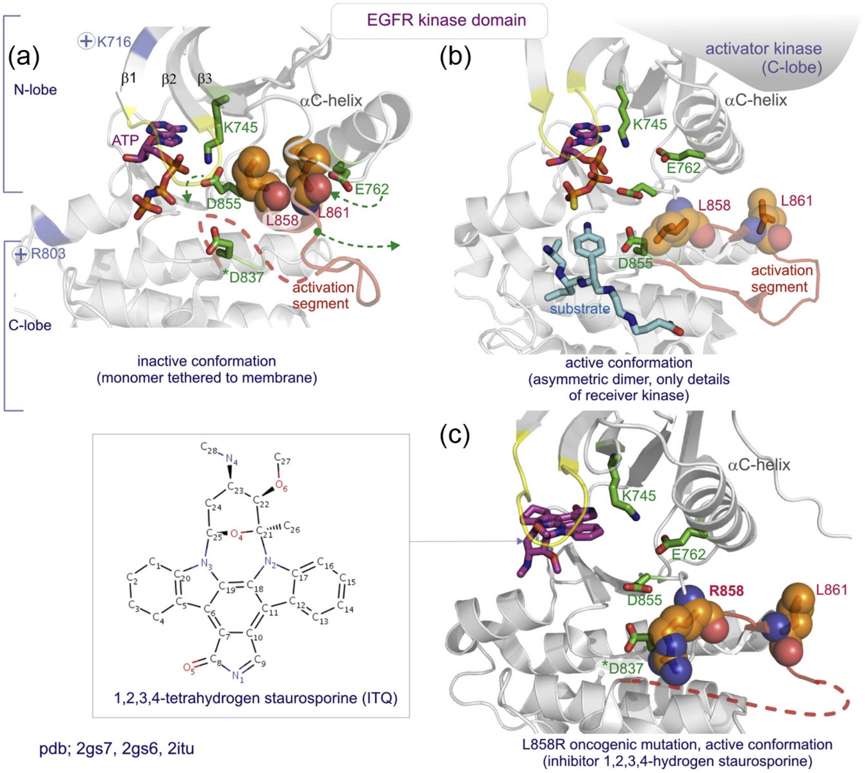
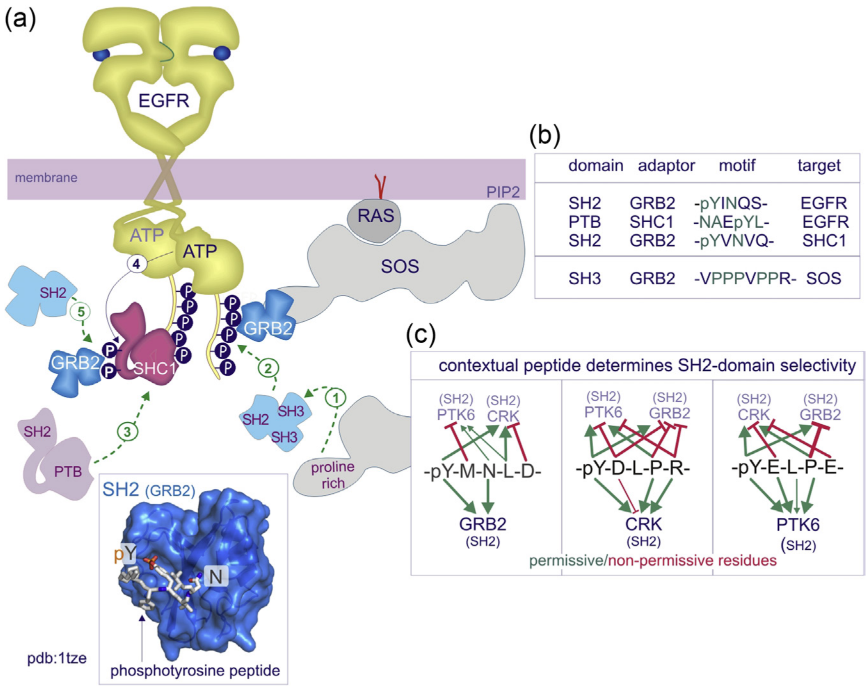


FIGURE 10-7 The EGFR receptor leucine wedge regulates kinase activity. (a) In the inactive conformation, the leucines 858 and 861, which are part of the activation segment, wedge into the N-lobe and distort the position of the αC-helix. The inactive kinase tethers to the plasma membrane with positively charged residues such as K692 and R779 (in blue). (b) The C-lobe of the activator kinase pushes the αC-helix (of the receiver kinase) inward, forcing the two leucine residues to move outward. The essential glutamate (E762) now effectively contributes to the coordination of ATP, the N-lobe shifts slightly downward and the kinase turns catalytically competent, ready to phosphorylate substrate. (c) Replacing L858 with a positively charged arginine brings about the same active conformation as observed in an asymmetric dimer. In this example, the ATP-binding pocket is occupied by 1,2,3,4-tetrahydrogen staurosporine (an inhibitor). The structure of the ligand 1,2,3,4-tetrahydrogen staurosporine (ITQ) is obtained from the Protein Data Bank. The residues of the human EGFR are numbered according to the sequence in UniProtKB (with inclusion of the signal peptides, 1–24).

EGFR mutations prevail in nonsmall cell lung cancer and they arise independent of smoking history. The cancer is treated with Erlotinib (Tarceva®, nucleotide analog that replaces ATP) or Cituximab (Erbitux®, antibody directed against the extracellular segment of the EGFR), together with classic chemotherapeutic agents such as platinum analogs and taxanes (Zarogoulidis et al., 2013).



domain	adaptor	motif	target
SH2	GRB2	-pYINQS-	EGFR
PTB	SHC1	-NAEpYL-	EGFR
SH2	GRB2	-pYVNVQ-	SHC1
SH3	GRB2	-VPPPVPPR-	SOS

FIGURE 10-8 Formation of receptor signaling complexes. (a) The phosphotyrosine residues of the active EGFR bind to proteins that either carry an SH2 or a PTB domain. As a consequence adaptors and effectors (enzymes) are recruited to the plasma membrane. The figure shows GRB2-mediated recruitment of the guanine-exchange factor SOS. SHC1 is also an adaptor and it recruits yet another adaptor, GRB2 which may then recruit SOS. The insert shows the structure of the SH2 domain of GRB2 attached to a small phosphopeptide. The phosphotyrosine in 1st position and the asparagine in 3rd position penetrate into SH2 and make important electrostatic interactions. (b) Examples of peptide sequences in the EGFR and the adaptor protein SHC1 that are recognized by SH2 or PTB domains. The SH3 domain of GRB2 binds to the proline-rich sequence of SOS. (c) Recognition of phosphotyrosines is determined by the presence of four other amino acids (contextual peptide). For instance, the sequence –pYMNLD is recognized by the SH2 domain of GRB2 but not (or weakly) by the SH2 domain of PTK6 or CRK because of the presence of non-permissive residues such as methionine in 2nd position for SH2–PTK6 and aspartate in 5th position for SH2–CRK. *(Information from* Liu et al. (2010)*.)*

Web resource

For more information about inhibitors and other protein-bound structures (with references made to the Protein Data Bank) consult: http://pubchem.ncbi.nlm.nih.gov. Enter for instance: "1,2,3,4-tetrahydrogen staurosporine" or "erlotinib."

Measuring assembly of receptor signaling complexes

The formation of signaling complexes and the subsequent signal transduction events have been studied in a number of ways.

1. By measurement of enzyme activity, the generation of second messengers, and analysis of tyrosine-phosphorylated substrates.
   The activated receptors for EGF and PDGF stimulate PLCγ. This results in the generation of DAG and IP3 and leads, within seconds, to the activation of protein kinase C and a rise in the concentration of intracellular free Ca^{2+} (Pandiella et al., 1988; Gilligan et al., 1990; Gonzalez et al., 1988). All of these can be measured. Furthermore, PLCγ itself becomes phosphorylated on tyrosine residues indicating that it interacts directly with the catalytic domain of the receptor. Activation of the receptors for PDGF and insulin also causes activation of phosphatidylinositol 3-kinase (PI3-kinase) (Higaki et al., 1996). This phosphorylates $PI(4,5)P_2$ forming $PI(3,4,5)P_3$, a signaling lipid (discussed in Chapter 16, "Signaling through the Insulin Receptor"). In addition, a number of serine/threonine kinases also become activated. These include ribosomal S6 kinase (implicated in protein synthesis), C-Raf kinase (see below) and the mitogen-activated protein kinases (MAPKs, see below). Most importantly, Ras becomes activated (Liu and Pawson, 1991; Medema et al., 1993; Muroya et al., 1992).
2. Co-immunoprecipitation of proteins with activated receptors.
   To investigate the specific interactions of activated receptors, cells pre-labeled with ^{35}S-methionine are stimulated and then solubilized with detergent. The receptors, together with any associated proteins, are precipitated using an antireceptor antibody. The associated proteins are detected by gel electrophoresis and autoradiography. Identification is achieved by microsequencing, immunoblotting, and other techniques. This was how the associations of protein tyrosine kinase receptors with Ras-GAP, PLCγ, and PI3-kinase were originally demonstrated (Meisenhelder et al., 1989; Koch et al., 1991). Using a similar approach, but with cell lysates and purified receptors, it was shown that a subunit (p85) of PI3-kinase binds to the PDGF receptor (Escobedo et al., 1991).
3. By studying protein associations in a cell-free system: cloning of receptor targets.
   Proteins expressed by a lambda-phage library in a bacterial host are screened for binding to the cytoplasmic domain of a receptor, labeled with ^{32}P-phosphate. The relevant bacterial clones are identified using autoradiography and the DNA sequence of the phage insert is determined. This was the way in which the

continued

associations of the EGF receptor with the adaptor Grb2 and the p85 subunit of PI3-kinase were discovered (Skolnik et al., 1991).

4. **By studying protein associations in protein microarrays.**
   Using this large screen approach it became apparent that binding of phosphopeptides occurs to SH2 or PTB domains that would not otherwise have been predicted according to the consensus sequence of the phosphopeptides. Furthermore, it became apparent that increasing the phosphopeptide concentration (the equivalent of increasing the expression levels of cell surface receptors) augments the number of different SH2/PTB domains that are recognized. This suggests that high expression of receptors provides the means to regulate not only the intensity of the signal but also the range of effector proteins that are activated (Jones et al., 2006).
5. **By studying protein–protein interaction in a yeast two-hybrid assay.**
   Using the yeast two-hybrid method it was shown that the insulin receptor substrate 1 (IRS-1) binds through its PTB domain to a phosphorylated tyrosine residue present in the juxtamembrane region of the receptor (O'Neill et al., 1994). The two-hybrid assay was also instrumental in showing that HRAS interacts directly with a conserved 81 residue segment at the N-terminus of the serine/threonine kinase BRAF (O'Neill et al., 1994).

PROTEIN DOMAINS THAT BIND PHOSPHOTYROSINES AND THE ASSEMBLY OF SIGNALING COMPLEXES

The assembly of receptor signaling complexes depends on protein–protein interactions that involve phosphorylated growth factor receptors and adaptor/effector proteins that bear SH2 or PTB domains. The first clues to the mechanism of interaction came from studies of p47$^{gag-crk}$ a transforming protein devoid of any catalytic activity (Matsuda et al., 1990). Its transforming activity relies on the presence of a motif similar to a conserved region of Src (and many other tyrosine kinases), designated Src homology 2 (SH2). This motif was found to interact with tyrosine-phosphorylated proteins (Sadowski et al., 2006). Indirect evidence for a role of SH2 domains in interactions with phosphorylated growth factor receptors emerged with the observation that all proteins that operate downstream of these receptors, or that are involved in cell transformation, possess one or two copies of this domain. Significantly, PLCγ, the only isoform directly activated by the EGF receptor, possesses two SH2 domains (Figure 10-9). Further evidence was provided by the finding that SH2 domains bind to phosphotyrosines present in activated EGF receptors (Moran et al., 1990).

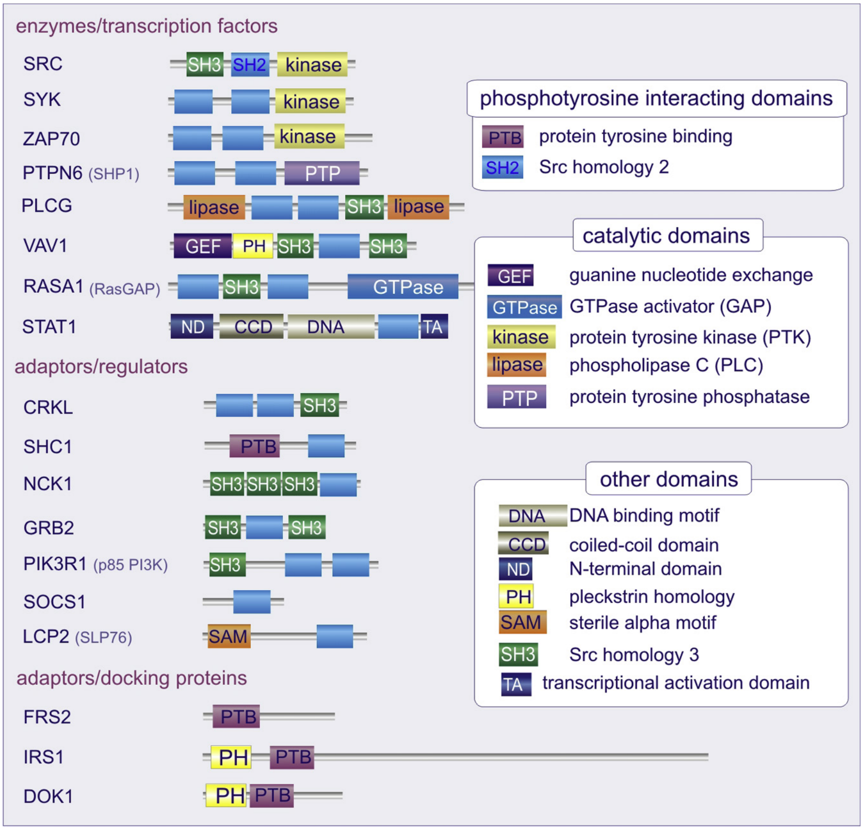


FIGURE 10-9 Domain architecture of proteins that associate with phosphorylated tyrosine kinase-containing receptors. Proteins that associate with tyrosine-phosphorylated receptors contain SH2 domains or PTB domains that recognize specific amino acid stretches around phosphotyrosine residues. Most of the effectors and adaptors and all docking proteins are substrates of the receptor kinases. Phosphorylated-docking proteins offer novel tyrosine phosphate-docking sites for yet other adaptors and effectors. Many of the proteins presented in this figure are discussed elsewhere in this book.

Another domain, structurally unrelated, that binds to phosphotyrosines, also present in many signaling proteins, is the PTB (phosphotyrosine-binding) domain. PTB was first identified in the adaptor protein SHC1, which also contains an SH2 domain (Kavanaugh and Williams, 1994; Blaikie et al., 1994; van der Geer and Pawson, 1995). These binding domains (SH2 and PTB1) recognize phosphotyrosines within specific oligopeptide motifs (the so-called contextual peptide). The selectivity varies both within the SH2 domains and between SH2 and PTB domains. It should be remembered that SH2 domains are recognized by their fold and global sequence but they nevertheless carry protein-specific sequence variations. In Figure 10-8 we illustrate how permissive and non-permissive amino acids determine which specific SH2 domain binds what (Liu et al., 2010). The human genome encodes 109 proteins having SH2 domains and 44 with PTB domains (Jones et al., 2006). Some of these are lined up in tandem, two SH2 or two PTB (see Figure 10-9). Most are either adaptors having no catalytic activity, or effectors such as phospholipases and protein/lipid kinases; others are transcription factors.

The adaptor protein SHC1 binds through its PTB domain to phosphorylated receptors and, on phosphorylation, becomes an indirect docking site for GRB2 (as opposed to the direct binding of GRB2 to the phosphorylated receptor). It is possible that yet other adaptor/docking proteins will come to light but the principle of action remains the same. Their roles may vary between cell types or may depend on the stimulus and its timing. It may also depend on the number of receptors, the concentration of the ligand, and the cytosolic concentration of the different adaptors and effectors.

Selectivity enables receptors to signal through panels of SH2- or PTB-containing proteins of which a nonexhaustive list is shown in Figure 10-4. Significantly, for the EGFR and ERBB2 (but not ERBB3), the interaction between phosphopeptides and target SH2 or PTB domains becomes less selective with increase in concentration (in the assay) of the phosphopeptides (Jones et al., 2006). What this means is that excess of expression of the EGFR or ERBB2 as occurs in for instance certain breast cancers may broaden the possible range of downstream signaling pathways. In other words, the growth factor signal not only becomes stronger, but also becomes more diverse.

The affinity (k_D) of SH2 or PTB domains for tyrosine-phosphorylated peptides is not particularly strong, of the order of 2 μM. In comparison, the affinity of growth factors for their receptors typically have $k_D \sim 0.1$ μM. As a consequence, the interactions have a half-life of less than 10 s, so that within the duration of receptor responses (minutes), numerous adaptor and effector complexes form, detach, and reform (Jones et al., 2006). This dynamic interplay allows tyrosine phosphatases to intervene, removing the phosphoryl groups from the receptor tyrosine kinases (see Chapter 16, "Signaling through the Insulin Receptor").

Gag is a glycosylated antigen, the gene encoding the internal capsid of the viral particle.

Crk is a C10 regulator of kinase. This is an SH2 and SH3 domain-containing adapter protein and is implicated in pathogenesis of chronic myelogenous leukemia.

$p47^{gag-crk}$ is a good example of how viruses disrupt genes resulting in chimeric proteins. In this case, viral Gag sequences are fused to the cellular CrkL. The mammalian cells express the gene product once it is inserted into their genome.

BASIC OUTLINE OF THE YEAST TWO-HYBRID PROCEDURE

The yeast transcriptional-machinery (RNA polymerase and associated factors) requires an interaction with regulatory transcription factors that bind to DNA. An example is the transcription of the ectopic bacterial lacZ gene (which codes for β–galactosidase). This entails binding of the transcription machinery to the transcription factor GAL4. By separating GAL4 into segments that bind DNA and that bind the machinery, transcription of lacZ cannot proceed. However, it can be restored by creating an interacting link between the two segments. In the yeast two-hybrid system, this is achieved by provision of two fusion proteins. One of these, the so-called bait, comprises the DNA-binding GAL4 segment fused to the protein of interest (e.g., Ras GTP-binding domain). The other, the so-called prey, comprises the transcription-machinery binding GAL4 segment fused to fragments of proteins derived from an appropriate cDNA library. Hundreds of clones expressing different cDNAs are thus created. The yeast clones that exhibit β-galactosidase activity (easily detected) are selected and analyzed for the inserted cDNA (Oliver, 1997).

Adaptors are composed of protein recognition domains (SH2, SH3, PTB, etc.) and are devoid of catalytic activity. They act to bring components of signaling pathways into close proximity. Examples are GRB2 and SHC1. By definition, effectors are catalysts, GTPases, kinases, phospholipases, etc.

BRANCHING OF THE SIGNALING PATHWAY

THE RAS-MAP KINASE PATHWAY

A number of signal transduction pathways branch out from the EGF-receptor signaling complex (Figure 10-10). Two such branches, the RAS and STAT pathways, are described in the following paragraphs, and others are considered more extensively in other chapters: phosphatidylinositol 3 kinase (PI3-kinase) in Chapter 16, "Signaling through the Insulin Receptor", phospholipase C (PLCG) in Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors" and in Chapter 9, "Protein Kinase C in Oncogenic Transformation and Cell Polarity." We return to STAT in Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases."

The Ras signaling pathway

From the tyrosine kinase to Ras

For 40 years it has been known that infection of rats with murine leukemia viruses can provoke the formation of a sarcoma (Simons et al., 1967).

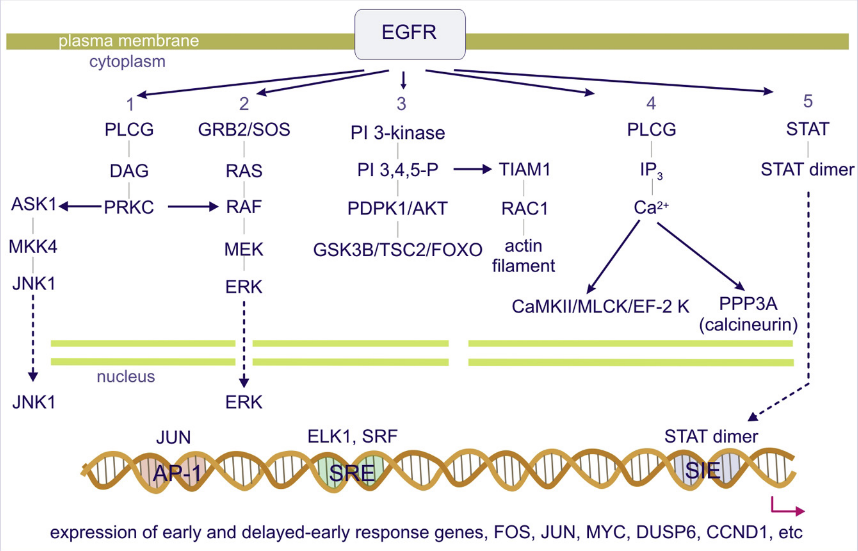


FIGURE 10-10 Branching of the signal transduction pathways. Following activation of a receptor protein tyrosine kinase, several signal transduction pathways can be activated. Five are indicated.

A major advance was the discovery that the Harvey murine sarcoma virus encodes a persistently activated form of H-Ras in which valine is substituted for glycine at position 12 (see Figure 10-19) (Sweet et al., 1984). Expression of this mutant in quiescent rodent fibroblasts results in altered cell morphology, stimulation of DNA synthesis, and cell proliferation. When overexpressed, (normal) H-Ras also induces oncogenic transformation as does microinjection of the mutant protein (Chang et al., 1982; Feramisco et al., 1984). Conversely, injection of neutralizing antibodies to inhibit normal Ras function reverses cell transformation (Feramisco et al., 1985). Stimulation of quiescent cells with serum or with growth factors promotes the binding of GTP to Ras (Satoh et al., 1990). It became apparent that Ras is an important component in the signaling pathways that regulate cell proliferation, but how this would fit into the known pathways emanating from growth factor receptors remained unclear for a considerable time. The first clues came from genetic analysis of signal transduction pathways that operate in the invertebrates *D. melanogaster* and *C. elegans*. Below we will describe how flies and worms contributed to the understanding of RAS-mediated growth factor signaling.

Sarcoma: (from the Greek for fleshy growth) is a cancer of the connective or supportive tissues (bone, cartilage, fat, muscle, blood vessels) and soft tissue.

Photoreceptor development in the fruit fly

The compound eyes of insects are formed of a hexagonal array of small units, ommatidia (in the case of the fruit fly, about 800 "small eyes"; return to Chapter 5, "Sensory Signal Processing," Figure 5-12). Each is composed of eight photoreceptor cells (R1–R8) and 12 accessory cells. On the basis of their morphology, order of development, axon projection pattern, and spectral sensitivity, the photoreceptor cells can be classified in three functional classes: R8, the first to appear, followed by R1–R6, and then R7. The photosensitive pigment resides in a stack of membranes, the rhabdomere. The larger rhabdomeres of cells R1–R6 are arranged as a trapezoid surrounding the rhabdomeres of cells R7 and R8, the R8 rhabdomere being located below R7 (Figure 10-11). The development of R7 requires the products of two genes: *sevenless* (*sev*) and *bride-of-sevenless* (*boss*). The phenotypes generated by loss-of-function mutations in either of these genes are identical, R7 failing to initiate neuronal development. These mutations are

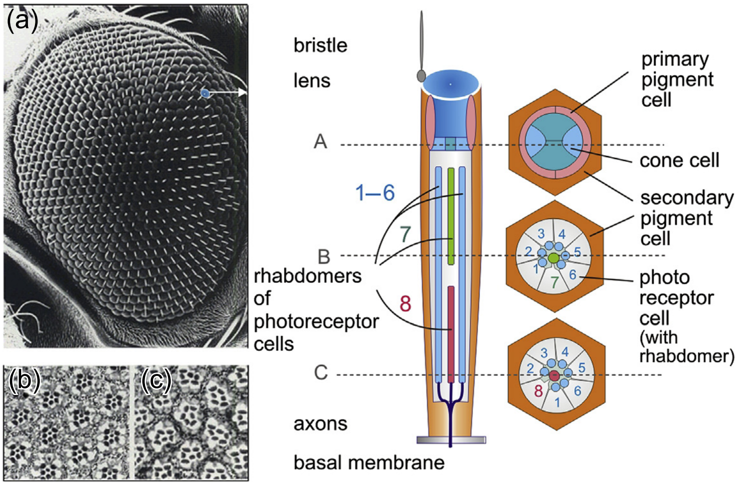


FIGURE 10-11 The *sevenless* mutation in fly eyes. The events leading to the development of cell R7 in eyes of *Drosophila* have provided a key to understanding the pathways initiated by receptor protein tyrosine kinases. Genes acting downstream of the *sevenless* receptor were revealed by screening for mutations that affect the development of cell R7 (green). (a) *Drosophila* compound with numerous ommatidia. (b) A thin section of the upper segment of an ommatidium and analyzed by electromicroscopy shows the arrangement of the rhabdomers (the stacked membranes of photoreceptor cells). (c) A similar section taken from a fly having the *sevenless* mutation, where the ommatidium only harbors six rhabdomers (R7 is missing). The drawing on the right illustrates the basic anatomy of a single ommatidial unit in longitudinal section. Sections cut at A, B, and C show the arrangement of the photoreceptor cells with their rhabdomers and pigment cells. Since two of the cells—R7 and R8 do not extend the full length of the ommatidial unit, the transverse sections B and C only reveal seven, not all eight cells. *(Picture adapted from Dickson and Hafen (Harris et al., 1976).)*

readily detected in a behavioral test. Given a choice between a green and a UV light, normal (WT) flies will move rapidly toward the UV source. Failure to develop cell R7, the last of the photoreceptor cells to be added to the ommatidial cluster, correlates with the lack of this fast phototactic response, and the flies move toward the green light (Harris et al., 1976).

While the *sev* product is required only in the R7 precursor, boss function must be expressed in the developing R8. Cloning revealed the boss product as a 100 kD glycoprotein having seven transmembrane spans and related to the metabotropic receptors (Hart et al., 1990). Although ultimately expressed on all of the photoreceptor cells, at the time that R7 is being specified it is only present on the oldest, R8 (Kramer et al., 1991). The product of the *sev* gene is a receptor protein tyrosine kinase (member of the insulin receptor family) (Hafen et al., 1987). Evidence for direct interaction between the products of these two genes came with the demonstration that cultured cells expressing the *boss* product tend to form aggregates with cells expressing *sev*.

It is now understood that the binding of boss (the ligand) to *sev* (the receptor tyrosine kinase) leads to the activation of kinase activity and that this ultimately determines the fate of R7 as a neuronal cell. Since a reduction in the gene dosage of the fly Ras1 impairs signaling by *sev*, and persistent activation of Ras85D obviates the need for the boss and *sev* gene products, it follows that the activation of Ras85D is an early consequence of *sev* activity (Fortini et al., 1992).

Further genetic screens of flies expressing constitutively activated *sev* led to the identification of *drk* and *Sos* as intermediate components of this pathway (adaptor proteins). The Sos protein shows substantial homology with the yeast *Cdc25* gene product, a guanine nucleotide exchange catalyst for Ras (Jones et al., 1991). While reduction in the gene dosages of *drk* and *Sos* impairs the signal from constitutively activated *sev*, there is no effect on signaling from constitutively activated Ras85D. Thus, in the pathway of activation, this places the functions of the *drk* and *Sos* products into positions intermediate between *Sev* and Ras (Figure 10-13). The *drk* gene codes for a small protein that consists exclusively of Src-homology domains, two SH3 flanking a single SH2 domain. Being devoid of catalytic activity, *drk* acts as an adaptor. It binds to the tyrosine-phosphorylated receptor and links it to the proline-rich domains of Sos (Lowenstein et al., 1992).

NEMATODES

More that 20,000 individual species of round worm in the phylum *Nematoda* (from the Greek, meaning thread like) have been described, though it has been suggested that there might be more than 500,000.

continued

NEMATODES *(cont'd)*

They inhabit all terrains, sea and fresh water, the polar regions, the tropics, and deep oceanic trenches. A large proportion is parasitic, including pathogenic forms that affect plants and animals.

The hermaphroditic *C. elegans* is a free-living roundworm, about 1 mm in length which inhabits temperate soils. It is used extensively as a model organism. The entire genome has been sequenced and the developmental fate of every one of the 959 somatic cells is known. When crowded or starved of food, *C. elegans* enter a larval stage called the dauer state. Dauer larvae are resistant to stress and do not age.

Neuregulin 1 (NRG1) and its receptors ERBB3 and -4 are implicated in the pathophysiology of schizophrenia. Among NRG1 receptors, ERBB4 plays crucial roles in neural development and in the modulation of NMDA receptor signaling. In the pre-frontal cortex of schizophrenic individuals, there is a marked increase in NRG1-induced activation of ERBB4 though their expression appears unaltered. NRG1 stimulation suppresses NMDA receptor activation and this is more pronounced in schizophrenia subjects (Hahn et al., 2006).

Vulval cell development in worms

In the nematode *C. elegans*, a similar pathway of activation involving autophosphorylation of a tyrosine kinase receptor leads to activation of the GTPase Let-60, a homolog of Ras (column two of Figure 10-13). This determines the development of vulval cells (Figure 10-12). Again, these proteins were first identified from genetic analysis of lethal mutations (let, lethal mutants), morphological changes in vulval development (sem, sex muscle mutants), or alterations in cell lineage (lin, lineage mutants) (Kornfeld, 1997). They constitute the components of a signal transduction pathway based on lin-3 (a product of the anchor cell), let-23 (a tyrosine kinase receptor of the p5 cell), and Sem-5 that associates with a (Sos-like) guanine nucleotide exchange protein. This brings about nucleotide exchange on let-3: lin-3 and let-23 are, respectively, members of the neuregulin (ligand) and ERBB (receptor) family.

In both worms and flies, the Ras protein acts as a switch that determines cell fate. In *C. elegans*, the activation of Ras determines the formation of vulval as opposed to hypodermal (skin) cells. In *Drosophila* photoreceptors,

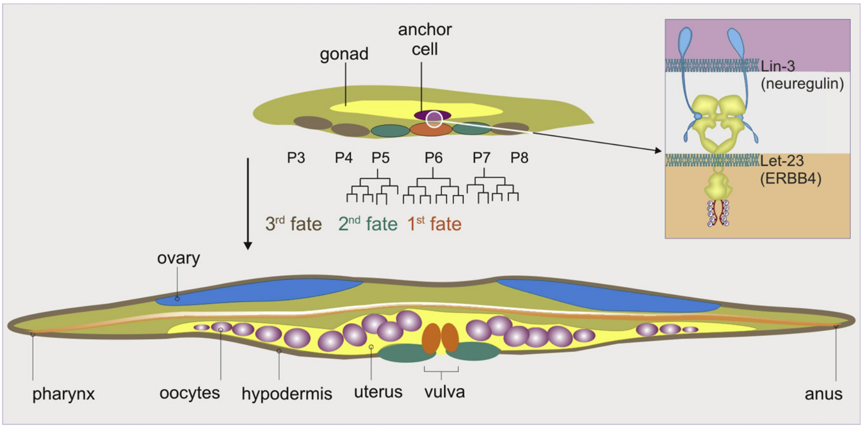


FIGURE 10-12 Vulval development in *C. elegans*. Because it is a relative simple structure, formed from just a few cells, the vulva is well suited for the genetic analysis of cell differentiation during embryological development. It is the product of just three cell lineages, the descendents of cells p5, p6, and p7. Development is initiated by a signal from the anchor cell that lies adjacent to p6. The ligand, lin-3 (homolog of neuregulin), produced by the anchor cell binds its receptor let-23 (homologous to members of the ERBB family) on the surface of cell p6. Cell p6 in turn releases signals to its neighbors, p5 and p7 and initiates a sequence of events involving the MAP-kinase pathway that determines the fate of these cells as components of vulval tissue.

the activation of Ras determines the development of R7 as a neuronal as opposed to a cone cell. In both cases, Ras proteins operate downstream of receptor tyrosine kinases that are activated by cell–cell interactions.

Web resources

For further information about *Drosophila* development and its genes we suggest: http://flybase.bio.indiana.edu/For more information about Nematode development and its genes we suggest: http://www.wormbook.org/.

Regulation of Ras in vertebrates

The elucidation of the Ras pathway in vertebrates was based on the identification of proteins having sequence homologies with those present in *Drosophila* and *C. elegans* (column 3 Figure 10-13) (Gale et al., 1993; Stern et al., 1993; Li et al., 1993). Expression or microinjection of these proteins (and appropriate reagents such as peptides and antibodies) were used to restore or modulate the activity of this pathway in cells derived from

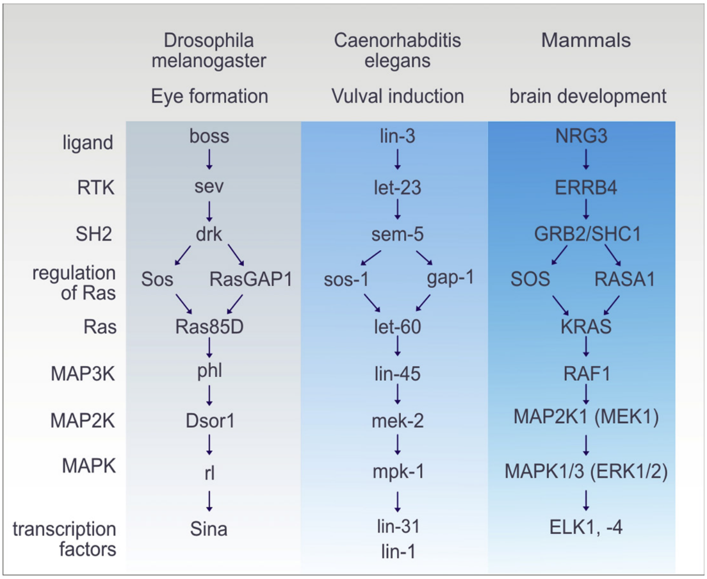


FIGURE 10-13 Comparison of signaling pathways activated by a receptor protein tyrosine kinase in species from different phyla. The striking homologies that exist between the proteins operating downstream of receptor tyrosine kinases in distant phyla has enabled the elucidation of the EGF receptor pathway. For the sequence in mammals, we mention neuregulin and its receptor ERRB4 as they are more closely related to boss and lin-3 compared with the EGFR. However, the same sequence applies for EGF acting on the EGFR.

mammals, flies, or worms, bearing loss-of-function mutations. A vertebrate protein GRB2, lacking catalytic activities but having SH2 and SH3 domains, was found to be capable of restoring function in sem-5-deficient mutants. In addition, GRB2 was found to associate with a protein that is recognized by an antibody raised against the *Drosophila* protein, Sos. In this way the sequence of events became apparent. GRB2 is an adaptor protein, linking the phosphorylated tyrosine kinase receptor to the guanine nucleotide exchanger in vertebrates. The mammalian Sos homolog, SOS1 is likewise a guanine nucleotide exchange factor which interacts with RAS (Aronheim et al., 1994). GRB2 is composed exclusively of SRC homology domains, one SH2 flanked by two SH3 domains, similar to the *Drosophila* adaptor protein *Drk*. Because of the nature of the interaction of SH3 with proline-rich domain, it is likely that GRB2 and SOS1 remain associated even under nonstimulating conditions. The main effect of receptor activation is to ensure the recruitment of the GRB/SOS1 complex to the plasma membrane (Rozakis-Adcock et al., 1993). Recruitment serves two purposes: firstly to facilitate the encounter with membrane-bound RAS and secondly to render the catalytic site active. SOS is a big protein composed of five domains of which CDC25 has

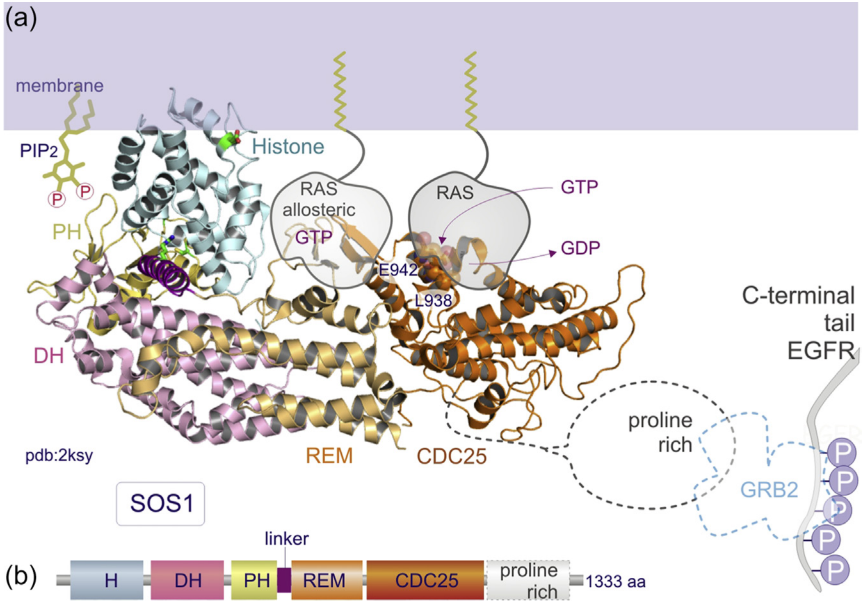


FIGURE 10-14 SOS structure function. The SOS protein, a guanine nucleotide exchange factor, is composed of multiple domains. Nucleotide exchange is catalyzed by the CDC25 domain through the action of E942 and L938 (which push GDP out of the nucleotide-binding pocket). SOS carries two binding sites for RAS: a RAS.GTP-binding site on the REM domain and a RAS.GDP-binding site on the CDC25 domain. RAS.GTP plays an allosteric role by enhancing the activity of CDC25. Access to the REM site is normally obstructed by the DH domain but this domain folds away when SOS is recruited to the membrane. Binding of both the histone and PH domain to the membrane plays a role in the change in conformation. The histone domain binds with a positively charged patch to the negatively charged membrane and the PH domain interacts with phosphatidylinositol-4,5-bisphosphate (PIP2). Membrane recruitment occurs through binding of GRB2 to the tyrosine phosphorylated growth factor receptors. GRB2 binds SOS via a C-terminal positioned proline-rich region.

nucleotide exchange activity. Paradoxically, efficient exchange requires the presence of RAS.GTP bound to the REM domain (Figure 10-14). The binding of allosteric Ras.GTP is normally obstructed by the DH domain, but this constraint is relieved when the histone and PH domain attach to the membrane. The histone domain binds with a positively charged patch to the negatively charged membrane and the PH domain specifically interacts with phosphatidyl-4,5-bisphosphate (PIP_2). Binding of SOS at the membrane initially results in a slow nucleotide exchange but a feed-forward mechanism sets in train when sufficient levels of RAS.GTP starts to occupy the allosteric binding sites (Boriack-Sjodin et al., 1998; Pierre et al., 2011; Margarit et al., 2003; Gureasko et al., 2008, 2010). Through this mechanism inappropriate RAS activation is reduced outside the membrane compartment and moreover only persistant growth factor signals will lead to substantial activation. Return to Chapter 2, "An Introduction to Signal Transduction," Figure 2-26, for more detail about the mechanism by which the CDC25 domain of SOS mediates nucleotide exchange.

Besides SOS, GAB1 is another GRB2-associated protein. GAB1 is a docking protein carrying numerous tyrosine residues that in turn may recruit yet other SH2- or PTB1-containing effectors. High-level expression of GAB1 enhances cell growth and facilitates oncogenic transformation (Holgado-Madruga et al., 1996).

The Ras-GTPase-activating protein, RasGAP (RASA1), also contains two SH2 domains and it too binds to phosphotyrosines on activated receptors. It is also a component of the signaling complex that assembles on activated PDGF receptors. It remains unclear what, if any, role this association of GTP-ase-activating protein with the receptor has in relaying signals. For instance, cells that express a mutant of the PDGF receptor that is unable to bind RasGAP manifest normal activation of Ras (Burgering et al., 1994).

From Ras to MAP kinases and the activation of transcription

The events following the activation of mammalian RAS lead to the activation of a series of kinases culminating in ERK (extracellular signal-regulated kinase). ERK was originally recovered as a serine/threonine phosphorylating activity present in the cytosol of EGF-treated cells and given the name mitogen-activated protein kinase, MAP kinase, or MAPK (Ray and Sturgill, 1987) (gene names MAPK1, -3, -4, -6, -7, and -15). They are serine/threonine kinases with a characteristic phosphorylation motif in the activation segment, an insert in the C-terminal lobe ("MAP kinase insert"), and a C-terminal extension that folds back on the N-lobe (Figure 10-15). Of the six isoforms, ERK1 and -2 contribute to the mitogen signal transduction pathway described here (see also Figure 10-27, "A family of MAP-kinase-related proteins"). Once activated, ERK can enter the nucleus to activate early response genes. All this operates quite independent of second messengers and, in some cell types, cyclic AMP actually opposes it (Dumaz et al., 2002). The earlier steps in the pathway involve the ordered activation of a three-tier system of protein kinases commonly indicated by MAPK3, MAPK2, and MAPK. Below we describe the order in the reverse way, following the path of discovery, starting at MAPK (Ahn et al., 1990).

The immediate activator of ERK is MEK (MAPK kinase-ERK Kinase). The MEKs comprise seven members (gene names MAP2K1–7) of which only MEKs 1, 2, and 5 are involved in the activation of ERK. MEKs are notable in that they act as "dualspecificity protein kinases," phosphorylating ERKs 1 and 2 both on a threonine and a tyrosine residue (Figure 10-15).

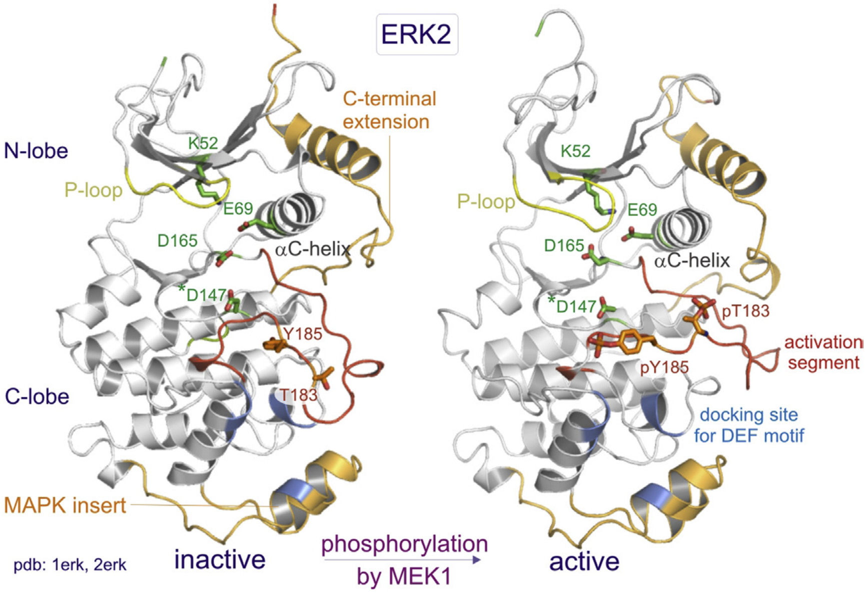


FIGURE 10-15 ERK structure and consequences of phosphorylation by MEK1. ERK has the characteristic kinase fold with two features that are specific for members of the MAP-kinase family: a kinase insert in the C-lobe and a C-terminal extension that folds back onto the N-lobe and makes multiple contacts with the αC-helix. Only in the case of members of the ERK subfamily (see Figure 10-27) does the kinase insert contribute to the formation of the FRS docking site for DEF motifs (which does not exist in other members) (Figure 10-20). Activation of ERK requires phosphorylation of the threonine (T183) and tyrosine (Y185) residue in the activation segment. This brings about an outward movement and allows the kinase to take on a "closed conformation."

These are present in the target sequence –L**T**E**Y**V–, which constitutes the activation segment. To date, the ERKs appear to be the unique substrates for phosphorylation by MEKs 1 and 2 indicating a particularly high level of specificity (Chen et al., 2001).

ERKs 1 and 2 (ERK1/2) are widely co-expressed. They appear to phosphorylate similar substrates at the same steps in the pathway, and they are similarly controlled. For instance, ERK1 knockout mice are viable but manifest defective thymocyte maturation (Pages et al., 1999) whereas the loss of ERK2 is embryonic lethal (Yao et al., 2003). In accordance with others, we will henceforth refer to ERK1/2. However, it should be noted that these kinases are not necessarily fully interchangeable.

MEK is the substrate of anthrax lethal factor, LF, one of several toxins produced by *Bacillus anthracis*. It resembles a metalloprotease. By cleaving the N-terminal region of MEKs 1 and 2, LF inhibits their kinase activity and prevents signal transmission through the ERK pathway (Duesbery et al., 1998). One of the consequences of MEK inhibition, which can be phenocopied with a chemical inhibitor, is increased vascular permeability leading to lung edema and hemorrhage (Bolcome and Chan, 2010). The lethality of anthrax is the consequence of a collective action of at least two other toxin proteins, protective antigen (PA) and edema factor (EF).

Upstream of MEK, the first kinase in the cascade is RAF (also described as MAP-kinase-kinase-kinase or MAP3K). Like RAS, RAF was initially identified as an oncogene product (v-raf) (Rapp et al., 1983). Three RAF kinases have been identified, ARAF, BRAF, and RAF1 (or C-RAF). They phosphorylate MEK at two serine residues in the activation segment giving rise to a catalytically competent kinase. The subsequent finding that activated RAS recruits RAF to the membrane and in consequence brings about kinase activation and links ERK with the RAS pathway (Moodie et al., 1993; Warne et al., 1993; Koide et al., 1993; Stokoe et al., 1994). Both the RAS-binding domain (RBD) and the cysteine-rich domain (CRD), present in the N-terminal region of the RAF kinases are instrumental for this function. The sequence of events is summarized in Figure 10-16.

RAF genes and RAF activation

The Raf oncogenes came to light as acquired oncogenes present in the murine retrovirus 3611-MSV (Rapp et al., 1983) and the avian retrovirus Mill-Hill 2 (Jansen et al., 1983). Homologs of these proteins are present in *Drosophila* (*phl*) and in *C. elegans* (*lin-45*). The mammalian genome contains three *raf* genes, of which RAF1 (also known as CRAF) is the most abundantly expressed and is present in all tissues. A- and BRaf are also widely expressed but, apart from neural tissues, at lower levels. The RAF proteins share a common architecture (Figure 10-17) comprising three main regions. The CR1 region contains the RBD and a CRD, both needed for membrane localization and activation. CR2 has a 14-3-3 protein-binding site and CR3 is the kinase domain (Wellbrock et al., 2004). Despite the fact that the different RAF proteins show much sequence similarity and all activate the same MEKs, mouse knockouts indicate that they are not functionally redundant.

Activation of RAF is complicated and still not clearly understood. It is associated with several proteins such as the serine/threonine phosphatase PP2A, heat shock proteins HSP90, the scaffold protein 14-3-3, and co-chaperone Hsp50/Cdc37 (Kolch, 2000). An essential feature is that the

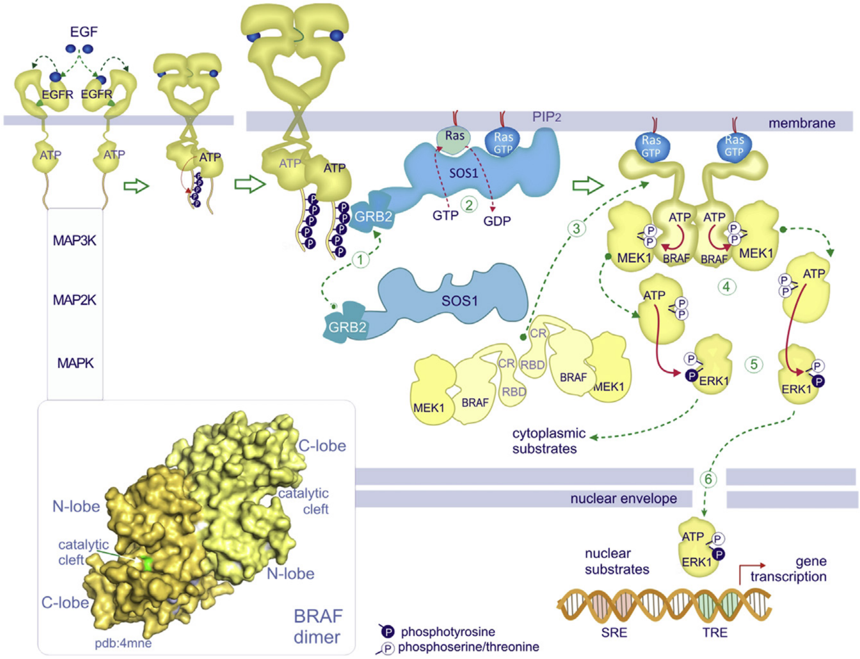


FIGURE 10-16 Regulation of the RAS–MAP-kinase pathway by the EGF receptor. (a) Upon receptor dimerization and *trans*-phosphorylation, the adaptor GRB2, in association with SOS1, attaches to the tyrosine-phosphorylated receptor through its SH2 domains. (b) This brings the GR2/SOS1 complex into the vicinity of the membrane to catalyze guanine nucleotide exchange on RAS. The exchange reaction is amplified by the growing presence of RAS. GTP which binds to an allosteric binding site. (c) The activated RAS (GTP) recruits a dormant BRAF/MEK complex to the membrane through interaction with the N-terminal RBD and CR domains. This relieves an auto-inhibitory constraint of the serine/threonine kinase BRAF and causes its subsequent activation through side-to-side dimerization. The insert shows the relative orientation of two BRAF-kinase domains without the presence of the regulatory segment (note that the two kinases associate in an upside down side-to-side fashion). Activation of BRAF leads to phosphorylation on two serines and activation of the dual specificity kinase MEK1 which in turn, after having left the complex, phosphorylates ERK1 on both a tyrosine and a threonine in the activation segment. ERK1 translocates to the nucleus there to phosphorylate transcription factors which bring about a change in the transcriptome. If the growth factor signal persists, the cell will engage in a cycle of division. The cascade comprises a three-tier system of kinases which occur in different version but always in the same sequence, starting with activation of a kinase in the position MAP3K (RAF), followed by MAP2K (MEK1), and ending with MAPK (ERK1) (shown on the left of the image). We mention that this is only one of the current scenarios because the events leading to activation of RAF are still disputed. SRE, serum response element. TRE, TPA-response element. For other abbreviations we refer to the text.

N-terminal region, comprising the CRD and RBD, hinders the activity of the catalytic domain. We distinguish two activation processes: the first comprising the removal of an inhibitory lock (Figure 10-18), the second a dimerization-mediated activation mechanism (Figure 10-17(b) and -19(b)). For RAF1 it was shown to be maintained in an inactive state by a locked conformation

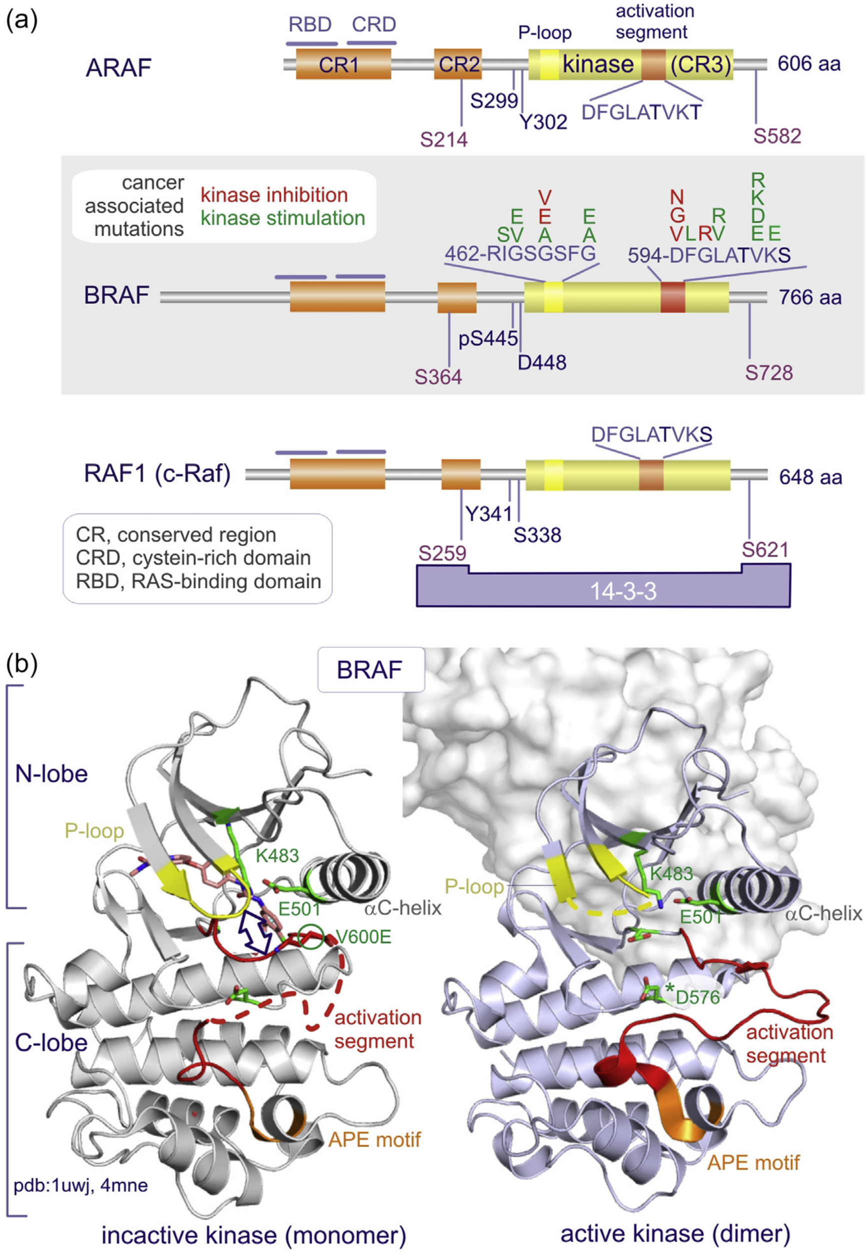


FIGURE 10-17 Domain architecture of the Raf proteins. (a) The Raf isoforms, A-, B-, and Raf1, share three conserved regions, CR1, CR2, and CR3 (kinase domain). CR1 contains the Ras-binding and the cysteine-rich domains (RBD and CRD, respectively), both necessary for membrane recruitment. CR2 contains one of the two 14-3-3 phosphoserine-binding sites, the other is in the C-terminal region. C3 represents the catalytic domain with

imposed by 14-3-3, tightly bound at two phosphoserine residues: S214 in the N-terminal segment and S582 in the kinase domain. The association of RAF with RAS.GTP at the plasma membrane requires dephosphorylation of a serine residue by PP2A (gene name, PPP2CA) (Ory et al., 2003) and this enables it to escape the inhibitory constraint of 14-3-3 (Figure 10-18). It also renders RAF1 susceptible to phosphorylation at other residues, especially in the negatively charged region adjacent to the kinase domain, but the kinase responsible remains elusive. BRAF lacks these N-region phosphorylation sites. All RAF kinases carry serine or threonine phosphorylation sites in the activation segment but, apparently, for BRAF their phosphorylation is not required for full activity (Rajakulendran et al., 2009). Essential to all this is that 14-3-3 remains attached to the regulatory segment (Hekman et al., 2004).

Once the inhibitory lock has been undone, the kinase remains inactive but is able to bind MEK in a face-to-face fashion (interaction at the level of the activation segments). Such dormant RAF/MEK complexes are detected in the cytoplasm (Haling et al., 2014). What is required for catalytic competence is a side-to-side dimerization of the RAF kinases, homo- or heterodimeric, but this is obstructed by their N-terminal tail. The 2nd activation step occurs when RAS.GTP binds the N-terminal segment and this allows two RAFs to find each other. As a consequence, the activation segment swings outward, the catalytic site becomes accessible to the two serine residues (S218 and S222) in the activation segment of MEK1 (Figure 10-17(b) and -19(b)). Phosphorylated and activated MEK1 detaches from the complex. What remains unclear in this model is whether or not the RAS–RAF complex has to fully dissociate in order to bind new MEK and then reactivate through dimerization?

◀ two phosphorylation sites in the activation segment (shown in dark blue in the sequence DFGLA…). Activation of A- and RAF1 requires phosphorylation of the serine and tyrosine residues in the acidic regulatory region between CR2 and CR3. Phosphorylation in the activation segment also occurs, but it is unclear whether or not this is vital for catalytic competence. Note that BRaf differs with respect to the acidic regulatory region. Tyrosine is replaced by aspartate (D448) and the S445 being constitutively phosphorylated. Of the mutations associated with cancer, shown in green and red, V600E in the activation segment is frequently observed. Note that both kinase stimulatory and inhibitory mutations have oncogenic potential. Figure adapted from ref (Wellbrock et al., 2004). We have numbered the residues in BRAF according to the sequence in UniProtKB. (b) Molecular structure of BRAF in its inactive and active states. The structure on the left is a V600E mutant kept inactive through an inhibitor BAY439006. It reveals that the P-loop interacts with the activation segment (indicated by arrow) and may thus play a role in keeping the kinase in an inactive state (and predicted to occur in the wild-type kinase without inhibitor). This explains why mutations in the P-loop control kinase activity. The image on the right represents the BRAF structure as it appears in a side-to-side dimer. The activation segment is swung to the outside and the P-loop has moved slightly upward. The (upside-down) kinase domain of the dimer partner of BRAF is represented in white surface.

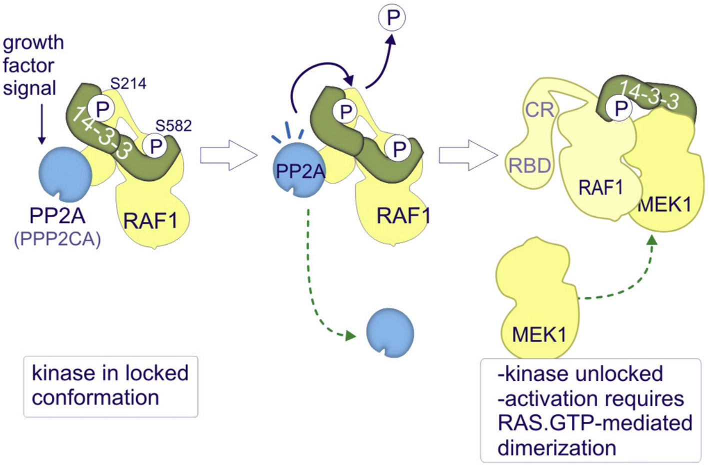


FIGURE 10-18 Unlocking RAF1. RAF1 is shown here in a locked and inactive conformation through binding of 14-3-3 to two phosphoserine residues, one in the N-terminal regulatory segment (S214 for RAF1) and the other in the kinase domain (S582). Growth factor signaling has to bring about the activation of a serine/threonine phosphatase (PPP2CA) that removes the N-terminal phosphate thereby relieving the lock. Activation of the kinase requires phosphorylation and importantly RAS.GTP-mediated dimerization (see Figures 10-16 and 10-17).

The Ras and RAF Oncogenes

Mutations in RAS occur in numerous cancers. Of all the RAS mutations detected, KRAS mutations are most frequent by far (86%), followed by NRAS (11%), and lastly HRAS (3%). In human pancreatic ductal adenocarcinomas, mutations in KRAS lie at the very origin of the cancer (constituting the “1st hit”) and they occur in 90% of the tissue samples analyzed. In colorectal cancer, mutations in KRAS constitute the “2nd hit” and they are detected in 50% of the tissue samples (Baines et al., 2011). Cancer-associated mutations are found in the highly conserved regions surrounding the nucleotide-binding pocket and they all lead to a reduction in GTPase activity (Figure 10-19). Examples are G12V, G13R, Q61R, or A146T. As a consequence the protein remains in a prolonged GTP-bound state thus amplifying the MAP-kinase pathway (among others). For more information about viral Ras, the discovery of monomeric GTPases and the GTPase cycle return to Chapter 2, “An Introduction to Signal Transduction,” Section “The monomeric GTPase family.”

Mutations in the BRAF (but not in A- or RAF1) have been detected in melanoma, thyroid, colorectal, and ovarian cancers (OMIM 164,757). There are more than 45 mutations of which the most common render the kinase constitutively active (gain-of-function mutation). Of these a valine to glutamate substitution (V600E) in the activation segment is prevalent

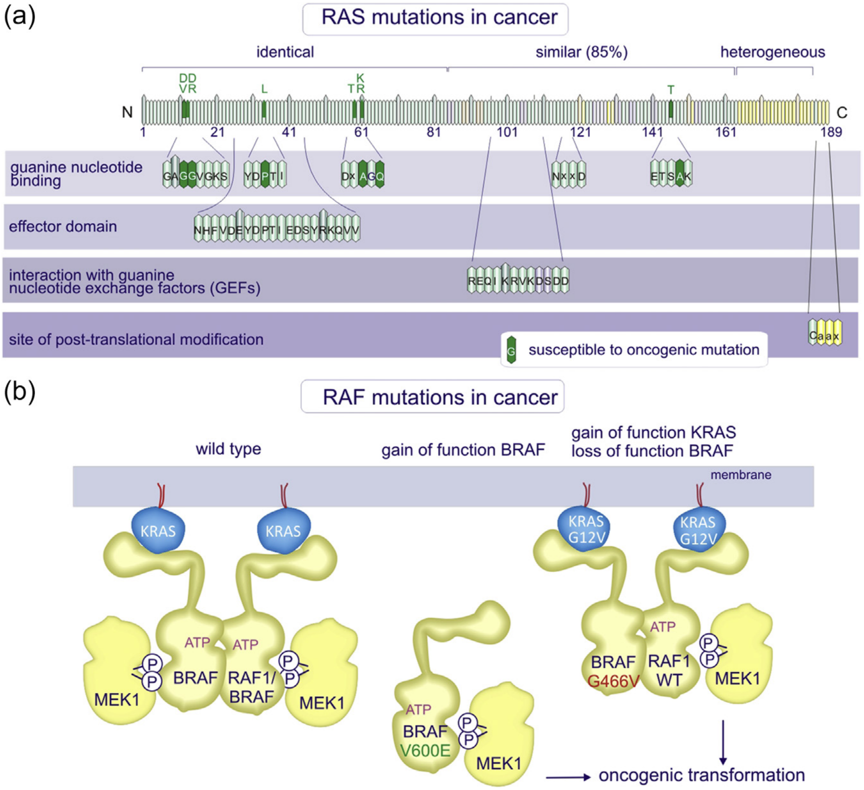


FIGURE 10-19 Mutations of RAS and BRAF associated with cancer. (a) Cancer-related mutations in RAS occur in the highly conserved motifs that surround the GTP-binding pocket. They cause a reduction in GTPase activity and thus increase the number of active RAS proteins, hence these mutations are referred to as gain-of-function mutations. (b) Cancer-related BRAF mutations come in two flavors, one occurring in the activation segment, V600E, the other in the P-loop G466V. The V600E causes the activation segment to swing outward and it renders the kinase catalytically competent without the requirement of dimerization. This leads to growth factor signal-independent phosphorylation and activation of MEK1. The G466V mutation, often found in nonsmall cell lung cancer, occurs in the GxGxxG motif of the P-loop and it renders the kinase inactive (a loss-of-function mutation). However, against the background of gain-of-function of KRAS mutations, it nevertheless boosts cell transformation by taking on the role of the "kinase-dead" activation partner of RAF1. For graphic simplification reasons, the BRAF dimers are not shown in opposite orientations (N-lobe bound by C-lobe of partner).

(Figure 10-19) (Davies et al., 2002). As a consequence, the activation segment folds away from the P-loop to facilitate access of substrate without further need for dimerization. In a MEK1 phosphorylation assay, the V600E mutation enhances activity 700-fold, and this gives rise to a 2–5-fold increase in the activity of ERK1 (Wan et al., 2004).

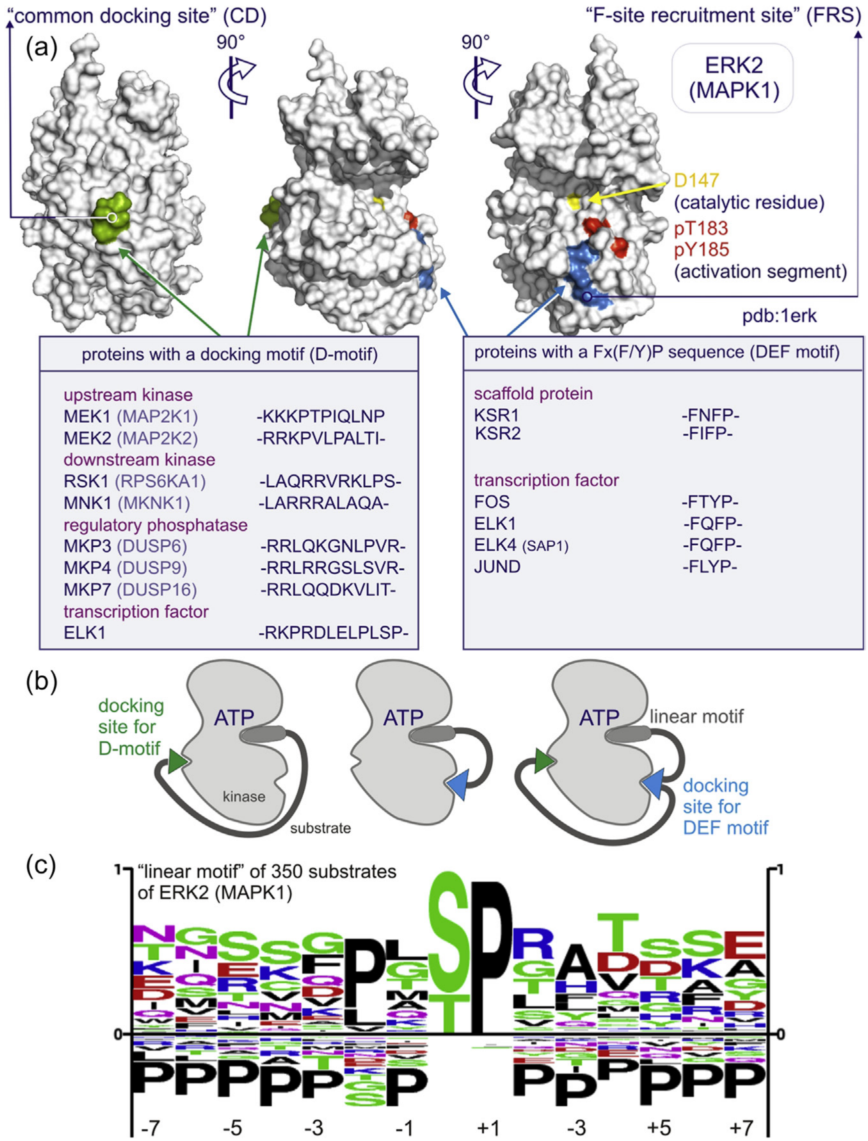
Paradoxically a loss-of-function (kinase dead) mutation, occurring in the P-loop (G446V) is also associated with cancer against the background of activating KRAS mutations (for instance G12V). The current idea is that the kinase-dead BRAF serves as an efficient activator of RAF1 (Heidorn et al., 2010) (Figure 10-17). The reason why BRAF but not ARAF or RAF1 are linked to tumor formation may be due to a difference in the N-terminal region. In BRAF, this carries an acidic sequence, and phosphorylation is not needed in order to confer a negative charge, as is the case for the other two. Fewer changes are needed to cause full deregulation.

Beyond ERK

Docking sites and an MAP-kinase phosphorylation motif

ERK1 and -2 (ERK1/2) phosphorylate substrates within the sequence -Ser/Thr-Pro- (Figure 10-20). Many proteins carry such a sequence, yet not all are ERK substrates so there must be other determinants of specificity. Many kinases and phosphatases are regulated by interactions with their substrates at noncatalytic regions, docking motifs, that help to avoid indiscriminate phosphorylations or dephosphorylations. The idea that MAP kinases form stable links with their substrates first arose with studies of JNK2 (gene name MAPK9), which was isolated from cell lysates in tight association with its substrate JUN. A stretch of positively charged residues surrounded by a zone of hydrophobicity in JUN, named docking or D motif, is required for the stable association (Kallunki et al., 1994, 1996). The same motif is also operational in substrate interaction with ERK1/2 and it recognizes a common docking site at the “back” of the kinase domain (Figure 10-20). The D motif occurs in upstream and downstream kinases, in phosphatases as well as in the transcription factors ELK1, ELK4, and JUND. Sequence swaps, grafting the docking domain of one member of the MAP-kinase family onto another cause a redirection of signals so that stress signals transmute into growth factor signals and vice versa (Tanoue et al., 2000; Brunet and Pouyssegur, 1996).

Members of the ERK subfamily of MAP kinases possess another docking site, just underneath the catalytic cleft, named the F-site recruitment site (FRS), and it recognizes proteins with an -Fx(F/Y)P- sequence (named the DEF motif). The “kinase insert” contributes to the composition of the FRS (return to Figure 10-15). Examples of proteins carrying such sequence are the scaffold proteins KSR1 and -2, and the transcription factors ELK1, ELK4, FOS, and JUND (Vinciguerra et al., 2004; Dimitri et al., 2005). The DEF motif seems to be reserved for substrates of the ERK family (not the other members of the MAP-kinase family).



proteins with a docking motif (D-motif)	
upstream kinase	
MEK1 (MAP2K1)	-KKKPTPIQLNP
MEK2 (MAP2K2)	-RRKPVLPALTI-
downstream kinase	
RSK1 (RPS6KA1)	-LAQRRVRKLPS-
MNK1 (MKNK1)	-LARRALAQA-
regulatory phosphatase	
MKP3 (DUSP6)	-RRLQKGNLPVR-
MKP4 (DUSP9)	-RRLRRGSLSVR-
MKP7 (DUSP16)	-RRLQQDKVLIT-
transcription factor	
ELK1	-RKPRDLELPLSP-

proteins with a Fx(F/Y)P sequence (DEF motif)	
scaffold protein	
KSR1	-FNFP-
KSR2	-FIFP-
transcription factor	
FOS	-FTYP-
ELK1	-FQFP-
ELK4 (SAP1)	-FQFP-
JUND	-FLYP-

FIGURE 10-20 Molecular surface of ERK2 showing docking sites. (a) ERK2 binds substrates that possess a D motif and interacts with the common docking site (CD, surface in green). Substrates that possess a DEF motif bind to a second docking motif on ERK, the F-site recruitment site (FRS, colored in blue), close to the activation segment (red spots). *(Adapted from Ref. Dimitri et al. (2005).)* (b) Schematic representation the different modes of interaction between ERK and its substrates: the linear motif, valid for all substrates, the docking site for proteins with a D motif, and the docking site for proteins with a DEF motif. Some proteins carry both D and DEF motifs. Docking sites increase efficiency and thus specificity of the phosphorylation reaction. (c) Sequence logo of ERK2 (MAPK1) substrates obtained from phosphosite analysis of 350 substrates. All phosphosites have a proline immediately downstream of a serine or threonine.

For a listing of MAP-kinase substrates, we suggest to explore PhosphoSitePlus and be stunned by the amount of information you have access to http://www.phosphosite.org/homeAction.do.

Enter for instance protein name: ERK1, then select organism: human. Underneath the Substrate Sequence Logo you click on "open substrate page." A long list of substrates appear in alphabetical order and each substrate has its own "protein page" with phospho-sequences and links to other kinases and signaling pathways as well as to relevant entry in UniProtKB.

Activation of protein kinases by ERK

Besides initiating transcription, to which we return later on, ERK1/2 also activates the MAP-kinase-activated protein kinases. This is a rather heterogeneous family of protein kinases that are grouped together because of a unique MAP-kinase phosphorylation motif, -LxTP- in the activation segment and a CaMK-like kinase domain (Figure 10-21). The RPS6A subfamily, so named because they have sequence similarity with the ribosomal S6-protein kinases (RPS6KB), carries a second catalytic domain in the N-terminal region which resembles that of protein kinase C with a characteristic "AGC-kinase C-terminal." Surprisingly, the activation segment of this N-terminal kinase domain is not a substrate of ERK1/2 so it is unclear how they are activated. It is possible that phosphorylation by ERK1/2 prepares the target kinase for a second, activating phosphorylation by the membrane-associated PDK1 (a key regulator of AGC protein kinases). Apart from listing the members of the MAP-kinase-activated kinases we will not discuss this subject further.

For a review on MAP kinases and their downstream protein kinases we refer to Roux and Blenis (2004).

Regulation of protein synthesis through the ERK–MNK1 pathway

ERK1/2 phosphorylates the eIF4E-binding protein (E4BP) and this causes its detachment from the eIF4E initiation factor. The pre-initiation complex (PIC) can now assemble around the 5′-cap (5′-m7G) of the mRNA (Sonenberg and Gingras, 1998). ERK1/2 also phosphorylate eIF4G and this brings about a reshuffle in the orientation of its HEAT domains allowing the protein kinase MNK1 (MKNK1) to bind (Dobrikov et al., 2013). MNK1 is next phosphorylated and activated by ERK1/2. MNK1 in turn phosphorylates eIF4E and this has an inhibitory effect on cap-mediated

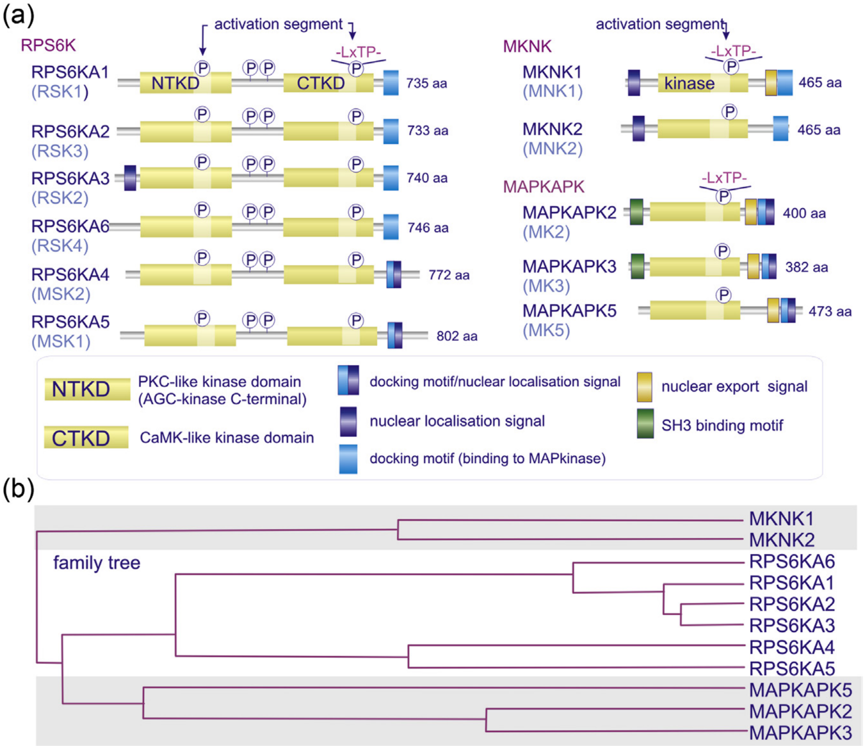


FIGURE 10-21 Domain architecture of the MAP-kinase-activated kinases. (a) The MAP-kinase-activated kinases come in three flavors, the RPS6, MKNK, and MAPKAPK, subfamilies. They share a common Ca^{2+}/Calmodulin-like kinase domain (CTKD) but with a unique phosphorylation motif in the activation segment (-LxTP-). Note that the RPS6 subfamily harbors a second kinase domain (NTKD), resembling protein kinase C with its characteristic AGC-kinase C-terminal. ERK1/2 do not phosphorylate the activation segment of the N-terminal kinase domain. (b) Sequence alignment of MAP-kinase-activated kinases reveals three subfamilies (family tree).

protein translation (Knauf et al., 2001). Importantly ERK1/2 phosphorylates the ribosomal p70 S6 kinase (RPS6KB1) and contributes to a more elaborate activation mechanism that is mainly driven by the TORC pathway which we shall discuss in Chapter 16, "Signaling through the Insulin Receptor" (Figure 10-22).

Activation of early response genes and the onset of the cell cycle

As a result of the double phosphorylation by MEK, ERK1/2 translocates to the nucleus by an as yet unclear mechanism. Not only does ERK itself not possess a nuclear localization signal (NLS), but also it is far from clear whether the proteins that bind ERK cause its nuclear localization. An alternative nuclear translocation motif has been discerned in the

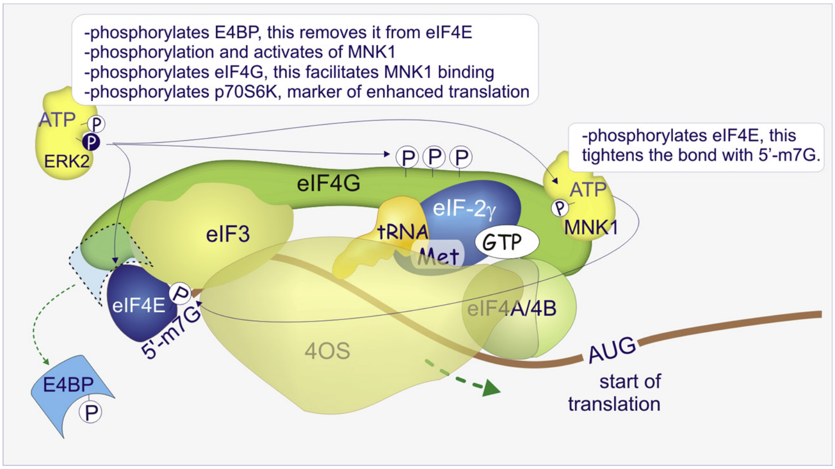

-phosphorylates E4BP, this removes it from eIF4E
-phosphorylation and activates of MNK1
-phosphorylates eIF4G, this facilitates MNK1 binding
-phosphorylates p70S6K, marker of enhanced translation
ATP
P
P
ERK2
P P P
-phosphorylates eIF4E, this tightens the bond with 5'-m7G.
eIF4G
ATP
P
MNK1
eIF3
eIF-2γ
tRNA
Met
GTP
eIF4E
P
5'-m7G
eIF4A/4B
40S
AUG
start of translation
E4BP
P

FIGURE 10-22 eIF-4E and the translation initiation complex. The initiation complex is formed around the eukaryotic initiation factor eIF-4E which is fixed to the 5′-m7G cap of mRNA. The initiation complex comprises tRNA^{Met} (first amino acid), numerous other eukaryotic initiation factors, and the small ribosomal particle 40S. ERK2 phosphorylates E4BP, which causes its removal from eIF4E. This is an essential step in the formation of a translation pre-initiation complex. ERK2 also phosphorylates eIF4G which leads to binding of MNK1. Upon phosphorylation and activation, MNK1 phosphorylates eIF-4E and this negatively controls the translation rate. Importantly, ERK2 contributes to the activation of p70S6K (RPS6KB), an event that coincides with enhanced translation.

C-terminal kinase insert comprising the sequence ser–pro–ser. Phosphorylation of this motif either by ERK itself or by casein kinase 2 (CSNK2A2) stimulates binding to importin 7 (IPO7), a protein involved in RAN-GTP-mediated active transport across the nuclear pore (Plotnikov et al., 2011). However, it should be noted that passive transport (simple diffusion) has also been demonstrated (Adachi et al., 1999). It is possible that the ERKs actually localize to the nucleus by default, but are continually returned to the cytosol by their interaction with MEK, present in both compartments and the bearer of an unmistakable nuclear export signal (Adachi et al., 2000). Nuclear ERK contributes to the expression of immediate early genes.

The early response genes are defined as genes that become transcribed as an immediate consequence of growth factor-mediated signaling events. Their activation is transient and can occur under conditions in which protein synthesis is inhibited. The gene products are generally detectable within 20 min of receptor stimulation. Activation of the EGF receptor causes rapid induction of FOS, one of the first growth factor-inducible transcription factors to be discovered (Kruijer et al., 1984). It occupies a central position in the regulation of gene expression of a second wave of genes, delayed early genes, such as MYC and JUN.

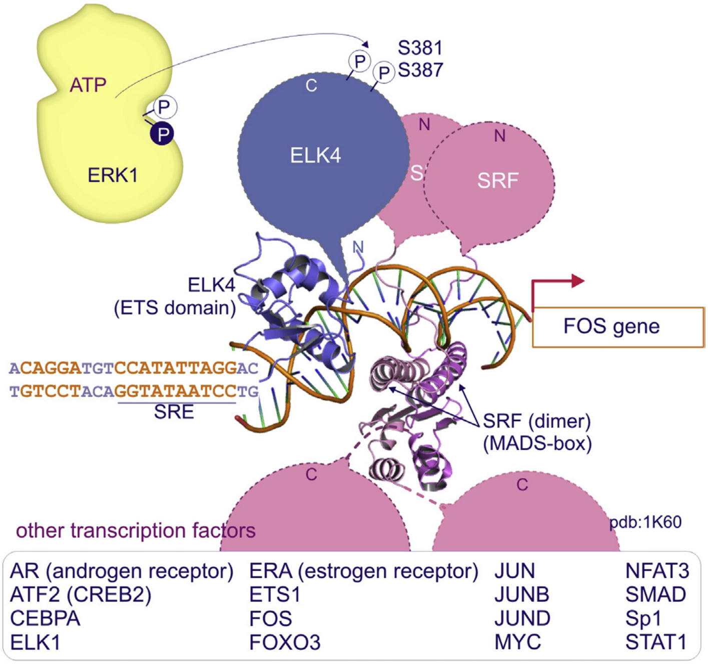


FIGURE10-23 Activation of transcription by ERK1. Within the nucleus, ERK1 phosphorylates ELK4 and transcription factor associated with two copies of the serum response factor (SRF) bound to the serum response element (SRE). It is unclear how phospho-ELK4 recruits transcription coactivators that pave the way to enhanced transcription of the FOS gene. A noncomprehensive list of other transcription factors phosphorylated by ERK1/2 is shown at the bottom of the image.

The promoter region of the FOS gene contains a serum response element (SRE), a DNA domain that binds two serum response factors (SRF), and one ELK4 (Figure 10-23) (Dalton and Treisman, 1992; Mo et al., 2001). ELK4 (also known as SFR-accessory protein or SAP-1) is phosphorylated by ERK1/2, on serine-381 and serine-387, but how this facilitates the recruitment of cofactors that stimulate transcription of the FOS gene is unclear (Figure 10-23) (Galanis et al., 2001). Importantly, upon growth factor treatment, the enhancer region of the FOS gene becomes rapidly and transiently phosphorylated and hyperacetylated at histone H3. It has been shown that the protein kinase MSK1 (RPS6KA5), activated by ERK1/2, phosphorylates histone H3 on serine-11 (the 1st methionine is included in the residue count) and could thus contribute to the chromatin remodeling that is necessary for activation of transcription (Vicent et al., 2006; Drobic et al., 2010). For more detail of histone modifications return to Chapter 2, “An Introduction to Signal Transduction,” Figure 2-22.

ELK4 has also been reported to suppress certain genes and this occurs through a phospho-ELK-4-mediated recruitment of SIRT7 (Kaikkonen et al., 2010; Barber et al., 2012). This protein, member of the Sirtuin family of proteins, specifically deacetylates histone H3 at lysine-18 (H3H18Ac) resulting in repression of transcription by rendering the enhancer region less accessible to the transcription PIC. The activity of the SIRT7 protein is associated with oncogenic cell transformation and plays a role in inducing anchorage-independent growth and escape from contact inhibition (Barber et al., 2012). For more information about the PIC, return to Chapter 7, "Bringing the Signal into the Nucleus," Section "Transcription and transcription factors" and Figure 7-10.

ETS FAMILY OF TRANSCRIPTION FACTORS

ETS, short for E−26 specific, was discovered as a transforming factor, v-ets, from acute avian leukemia retrovirus E26 (Leprince et al., 1983). This virus causes erythroblastosis in mice and myeloblastosis in chicken. Thereafter, mammalian homologs, initially named c-ets, came to light (ETS1 and ETS2) (de Taisne et al., 1984). Related ETS-domain-containing proteins (Ets-like) were also revealed, and one of them, ELK4, was first identified as part of a complex of three components together with two SRF proteins. It was therefore initially referred to as the SRF-accessory protein (SAP-1) (Dalton and Treisman, 1992). The human genome contains 29 ETS-domain-containing proteins with three ELK members that constitute the subfamily of Ternary Complex Factors or TCF.

FOS, from feline osteosarcoma virus.

MYC, the cellular counterpart of the transforming gene of the avian leukosis retrovirus MC29. MYC belongs to a small group of "master transcription factors" that play an essential role in cellular identity and in the control of cell proliferation (Oskarsson and Trumpp, 2005; Witmarsh and Davis, 2000). Return to Chapter 7, "Bringing the Signal into the Nucleus."

JUN, from avian sarcoma virus-17: we are informed that ju-nana is 17 in Japanese.

Expression of FOS brings about a second wave of transcription, the delayed-early genes, this time AP-1 driven and resulting in the expression of MYC and JUN among others. These too are substrates of ERK1/2 and their phosphorylation further boosts transcription of yet other genes

(Murphy et al., 2002, 2004). Examples of other transcription factors phosphorylated by ERK are presented in Figure 10-23. We return to ERK and its role in the regulation of SMAD in Chapter 17, "TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases."

With respect to the cell cycle, a key starting point is the expression of cyclin D, which, together with the kinase CDK4, drives progression through the early (G1) phase of the cell cycle (Whitmarsh and Davis, 2000). Cyclin D1/CDK4 phosphorylates and inactivates the retinoblastoma protein (RB1) and this removes a key transcriptional constraint for expression of enzymes involved in the replication of DNA. The retinoblastoma protein holds the cell in G1/G0 and therefore qualifies as tumor suppressor. Transcription of cyclin D1 is facilitated by ERK1/2 through induction of the transcription factors FOS, FOSL (Fra1), FOSL2 (Fra2), JUN, and JUNB, all components of the AP-1 complex that, when phosphorylated bind to the cyclin D1 gene promoter and drive its expression (Bakiri et al., 2000; Herber et al., 1994). ERK1/2 are also instrumental in the formation of stable cyclinD1/CDK4 complexes (Ladha et al., 1998) and finally, ERK1/2 also phosphorylate and inactivate the transcriptional co-repressor TOB1, that normally silences expression of CyclinD1 (Suzuki et al., 2002). These events set the stage for the expression of genes that are instrumental in the replication of the genome. Examples are genes encoding helicases, topoisomerases, DNA polymerases, and ligases.

In the case of the EGF receptor, the activation of ERK and its translocation into the nucleus is an absolute requirement for cell proliferation. Forced retention in the cytoplasm suffices to block growth factor-induced DNA replication (Pages et al., 1994). Weak signals that fail to induce cell proliferation also cause ERK1/2 to move into the nucleus, but it then recycles to the cytosol within 15 min. A strong mitogenic signal can ensure nuclear retention for up to 6 h (Kahan et al., 1992). Importantly, the signal must also be reinforced by signals from cell adhesion complexes (Aplin et al., 2001). We return to the question of cooperation between growth factor receptors and cellular adhesion in Chapter 11, "Signal Transduction to and from Adhesion Molecules."

The RB1 protein was first identified as the product of a gene that is deleted in patients with retinoblastoma, a tumor originating in the retina (Lee et al., 1987). The RB1 protein is a member of the family of nuclear pocket proteins that bind and inactivate the E2F transcription factor and prevent cells from entering S phase. Inactivation of the RB1 gene occurs in numerous other tumors. In the absence of RB1, cells enter into S phase more readily and they do not require the normal array of extracellular growth signals conveyed upon the expression and activation of cyclinD/CDK4 in order to proceed.

FINE TUNING THE RAS–MAP-KINASE PATHWAY: SCAFFOLD PROTEINS

We have explained that a sequential series of protein modifications determines the propagation of cell surface signals into the nucleus. A second level of regulation is presented by the interaction of scaffold proteins with components of this signaling cascade.

MAP-kinase scaffold proteins discovered in yeast

Signaling pathways are organized by protein kinases and phosphatases but also by accompanying proteins that have both a wiring and allosteric function. Wiring means that these proteins act as tethers, combining signaling partners in complexes and localizing them, by interacting with anchor proteins, to specific sites in the cell. These complexes warrant both specificity and efficacy. The allosteric part implies that protein–protein interactions play a role in protein kinase activation and in access to substrate. These accompanying proteins are referred to as scaffold proteins. It must be clear from all this that both the levels of expression of kinases and phosphatases as well as the scaffold proteins play an essential role in the outcome of signaling pathways both with respect to productivity and selectivity. How gene expression of the different components is coordinated is still largely unexplored.

The physiological relevance of scaffold proteins to MAP-kinase activation was first demonstrated in *Saccharomyces cerevisiae* (baker's yeast). Here, in the response to mating pheromones (ligands that interact with G protein-coupled receptor), STE5 acts as a scaffold that binds and links STE11 (a MAP3K), STE7 (a MAP2K), and FUS3 (a MAPK) to form a MAP-kinase signaling cassette (Choi et al., 1994; Elion, 2001; Lamson et al., 2006). In addition, STE5 also links the cassette to STE4 and STE18 (β and γ subunits of heterotrimeric G-protein, respectively) and to STE20 (equivalent to p21-activated kinase or PAK1) (Figure 10-24). Indeed STE5 has multiple kinase-binding sites and acts as a tether but it also plays a crucial role in rendering FUS3 a suitable substrate for STE7 (Good et al., 2009). As a consequence it drives the signal into the direction of a mating response rather than giving rise to filamentation (which occurs under conditions of starvation). This is not the whole story, STE5 harbors a second binding site for FUS3 and this leads to a feedback inhibition occurring more upstream (Bhattacharyya et al., 2006). Manipulating the composition of signaling complexes by creating chimeric scaffolds that recruit specific phosphatases or allosteric regulators was shown to affect the output of this MAP-kinase signaling cascade (Bashor et al., 2008). Such engineering experiments provide important information of how the flow of signaling event is controlled in vivo.

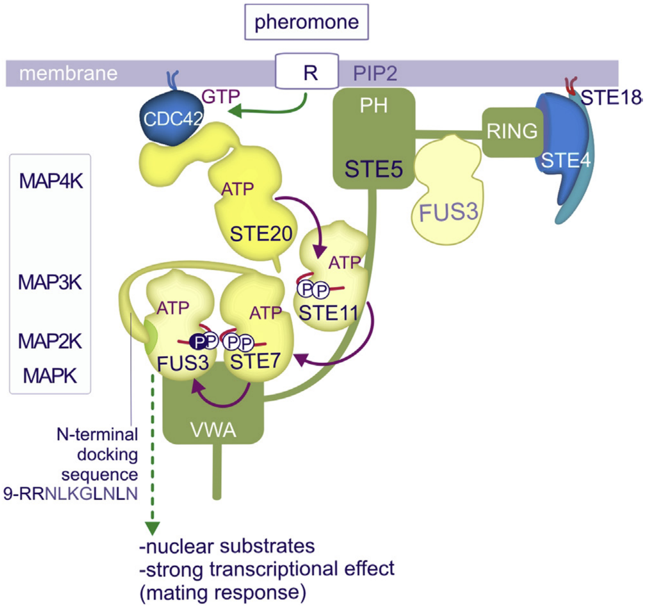


FIGURE 10-24 Yeast scaffold proteins and their role in pheromone signaling. STE5 (in green) assembles various MAP kinases (STE11, STE7, and FUS3) and brings them to the membrane by linking its RING domain to STE4/STE18 (βγ subunit) and its PH domain to phosphatidylinositol-4,5-bisphosphate (PIP_2). Here it encounters the activation complex comprising CDC42 (GTPase) and STE20 (S/T protein kinase, level of MAP4K). The signal passes, in the form of phosphorylation and activation, from STE20 to STE11 and then to STE7. In order to proceed the cascade, the VWA domain of STE5 plays a crucial role. STE7 docks with its C-terminal RRNLKGLNLN motif (return to figure to 10-20) onto the common docking site (CD) of FUS3. The interaction between the two is further strengthened by a mutual binding of the VWA domain of STE5. This not only brings the kinases together but also brings about a shape change of the activation segment which renders FUS3 a suitable substrate for STE7. Upon phosphorylation and activation, FUS3 initiates the mating response. Activated FUS3 has another binding site on STE5, in between the PH and RING domain, but here it blocks the passage of the receptor signal.

Mammalian scaffold protein that regulates MAP-kinase signaling

KRS

There are no animal homologs of STE5 but there are several other proteins that provide scaffold functions for the MAP-kinase cascades. An example is kinase suppressor of Ras, KRS, discovered in genetic screens performed in *Drosophila* and *C. elegans* (Therrien et al., 1995, 1996; Kornfeld et al., 1995). Its loss of function leads to impaired RAS–RAF–MEK signaling, hence its name. KSR is evolutionary conserved (two human homologs, KSR1 and -2), its site of action situates between RAS and RAF. Although the KSR proteins have a kinase fold and qualify among the tyrosine-kinase-like (TLK) kinases (Figure 2-36, Chapter 2, “An Introduction to Signal Transduction”), they lack the conserved lysine in the N-lobe that normally coordinates ATP, and as a consequence they only show minimal kinase activity. The KSR proteins mainly exert their action on the RAS–MAP-kinase pathway as scaffold proteins. Although not strictly required, their presence greatly enhances signaling through this pathway (Nguyen et al., 2002). The KRS protein has a similar domain organization as RAF and moreover their kinase domains have structural similarities. As a consequence KRS proteins can self-associate in a side-to-side dimer but they can also act as allosteric regulators by binding to RAF in a similar side-to-side fashion (as occurs in BRAF dimers, return to Figures 10-16 and 10-17). KSR2 bound to BRAF also interacts with MEK1 and this results in the phosphorylation of MEK1 on serine-24 and -72, albeit inefficiently. It does not phosphorylate the activation segment of MEK1, but changes its conformation so that it can be phosphorylated more easily by an “external” BRAF (Wimmer and Baccarini, 2010; Brennan et al., 2011). How this relates to the aforementioned MEK1 phosphorylation by BRAF dimers remains to be resolved. KSR1 and -2 have a docking site for ERK1/2 (two sequences, -FNFP- or -FIFP-) which recognized the F-site recruitment site.

In order to facilitate MAP-kinase signaling, KSR must be at the membrane and it can do so with its C1 domain (a Zn-finger motif also found in protein kinase C) which binds diacyglycerol. The diacylglycerol occurs in the membrane after activation of phospholipase C-γ (PLCG), one of the effectors of the EGFR (return to Figure 10-10). However, the C1 domain is associated with a protein that impedes mitogenic signal propagation (IMP; gene name, BRAP) (Matheny et al., 2004) and, in addition, the entire protein is sequestrated in the cytoplasm through an interaction with a 14-3-3 protein (Muller et al., 2001). In order for KSR to be operational it first needs to be dephosphorylated through the serine/threonine phosphatases PP2A (PPP2CA), the same phosphatase that dephosphorylates RAF (return to Figure 10-18). Then BRAP has to be removed and this occurs through degradation. BRAP contains a RING-H2 motif, characteristic of proteins that facilitate ubiquitinylation and it functions as an E3 ligase (see Chapter 13, “Activation of the Innate Immune System,” Figure 13-12). The intrinsic but dormant E3 ligase of BRAP is activated by RAS. GTP and this enables its ubiquitinylation and subsequent recognition by the proteasome. Now KSR translocates to the membrane and participates in the RAS–RAF–MEK signaling cascade (Figure 10-25). The importance of KSR1 in MAP-kinase signaling is further emphasized by the finding that mice lacking this scaffold protein are much less sensitive to oncogenic signaling through Ras (Lozano et al., 2003). We finish this paragraph by mentioning that loss-of-function mutations in KSR2 occur in people with severe early-onset obesity. As a consequence fatty-acid β-oxdiation and glycolysis are impaired giving rise to a low heart rate, low muscle tone, and hyperphagia (Pearce et al., 2013).

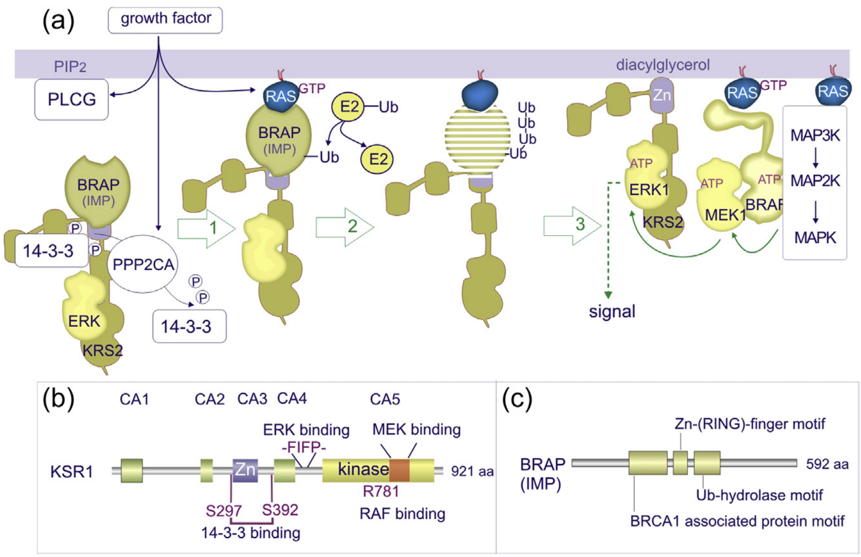


FIGURE 10-25 Dual effector interaction of RasGTP that leads to effective signaling to ERK. (a) In order to activate the ERK pathway, Ras has to not only recruit C-Raf, but also remove BRAP (IMP), the inhibitor which prevents formation of the RAF–MEK–ERK signaling cassette. RasGTP binds BRAP (1) and this initiates a series of ubiquitylations that mark the protein for destruction by the proteasome (2). ERK2, linked to the scaffold protein KSR1, now joins BRAF-MEK1 at the membrane, enabling the signal to pass from one kinase to another. (b) Conserved domains in KSR1. CA1, glycine-rich, CA2, proline-rich, CA3, cysteine-rich containing a Zn-finger which resembles the PMA/DAG-binding site (C1 domain) of protein kinase C, CA4, Ser/Thr-rich, CA5, a kinase domain that resembles that of Raf, but lacks an essential lysine and is therefore weakly active *(adapted from Refs Matheny et al. (2004) and Kolch (2005).)*. MEK is able to bind the activation segment of KSR1 whereas RAF binds in a side-to-side fashion involving arginine-781. ERK binds with its F-site recruitment site to the FIFP sequence. Not all three proteins can bind simultaneously. Phosphoserine-297 and -392 attach to a 14-3-3 protein and this sequesters KSR into the cytoplasm (excluded from the membrane). (c) The domain architecture of IMP reveals a Zn-(RING)-finger, involved in ubiquitylation. The gene name BRAP has been obtained because of the presence of a BRAC1-protein motif.

RKIP

RKIP is an inhibitor of the Ras-ERK pathway (Yeung et al., 1999). It binds to RAF1 (and weakly to MEK1, but not to BRaf) and it prevents MEK activation. When phosphorylated by protein kinase C, it is displaced from RAF1, relieving this inhibition and so reinstating the RAS-ERK pathway and contributing to gene transcription through the AP-1 complex (return to Chapter 9, "Protein Kinase C in Oncogenic Transformation and Cell Polarity," Figure 9-13) (Corbit et al., 2003) and return to Chapter 2, figure 2-52.

ARRB

After activation by their ligand G protein-coupled receptors bind β-arrestins (ARRB1 and -2) and these too act as scaffold proteins for the

MAP-kinase pathway. Unlike KSR1, the β-arrestins appear to retain active MAP-kinase signaling complexes in the cytosol and thereby prevent translocation and phosphorylation of nuclear substrates (Luttrell and Lefkowitz, 2002). Apart from binding MEK1–ERK1/2, β-arrestins also act as scaffolds for partners of the JNK pathway (MAPK3K5 (ASK1), MAP2K4 (MEK4), and MAPK10 (JNK3)) (Figure 10-27). Arrestins and their mode of recruitment by G protein-coupled receptors are discussed in Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," Figure 3-27.

LAMTOR3

MEK-binding partner 1 (MP1; gene name, LAMTOR3) localizes the MAP-kinase-signaling cassette to late endosomes through an interaction with the endosomal p14 (LAMTOR2) and p18 (LAMTOR1) adaptor proteins (Wunderlich et al., 2001; Teis et al., 2002). Signaling from the receptor continues in the endosomal compartment (Wiley et al., 2003; Schoeberl et al., 2002). The gene name LAMTOR3 originates from the fact that this protein also constitutes regulatory subunits in the MTOR protein kinase complex hence the double name: late-endosomal MAP kinase and MTOR activator 3. For more information about MTOR, see Chapter 16, "Signaling through the Insulin Receptor".

For a review that focuses on conceptual aspects of scaffold proteins we recommend: Good et al. (2011).

WHY ARE THE SIGNALING PATHWAYS SO COMPLICATED?

And why are there so many apparently redundant components? In fact, not all pathways are long and complicated. Some, like the pathway involving Notch (see Chapter 19, "Cell Fate Determination by Notch") or the STAT proteins (see below) are relatively straightforward, entailing only the modification of a signaling protein at the membrane followed by its direct translocation into the nucleus. More generally however, mechanisms tend to be complex and while there is no simple explanation, the reasons for complexity must stem from the need for cells to be able to sense multiple inputs, to commit to sets of appropriate responses, and to conduct them in controlled and precise ways. In general:

- Growth factor signals must initiate and synchronize both transcriptional and metabolic events. This requires multiple effectors, both nuclear and cytosolic.

- Signals need to propagate among and between different cellular compartments. (For example, receptors may be removed from the cell surface by endocytosis, processed in vesicular compartments and recycled back to the plasma membrane.)
- A single signaling protein can only make contact with a limited number of effectors.
- Signals must be amplified in order to exceed downstream threshold conditions and ensure effective responses.
- Signals must be robust and their propagation should not rely on a single critical component.
- A timely and measured response requires feedback circuits in order to maintain balanced and dynamic relationships between inputs and outputs.
- Signals from different inputs must converge at sites of integration that determine appropriate output signals.

Beyond all this, one must recall that signal transduction pathways were never designed, but are the result of numerous rather messy trial and error processes. In the end, these proved, and continue to prove, to be of benefit to the organisms of which they form a part.

TERMINATION OF THE ERK1/2 RESPONSE

The ability to attenuate and to terminate signals initiated by growth factors is crucial and there are numerous ways by which this is achieved. We limit the discussion to events affecting the receptors and the adaptors and effectors that bind to them.

First, there are the phosphatases, including the cytosolic PTP1B (PTPN1), SHP1 (PTPN6), SHP2 (PTPN11) and the transmembrane phosphatase DEP1 (PTRJ), that strip phosphates from phosphotyrosine residues of receptors, adapters, and kinases. ERK1/2 induce their own dual specificity phosphatases which are expressed in the wave of immediate early genes following growth factor treatment. Examples are MKP1 (DUSP1), operating in the nucleus and MKP3 (DUSP6) which functions in the cytoplasm. Finally, serine/threonine phosphatases, such as the already mentioned PP2A (PP2CA), dephosphorylate and deactivate all members of the large MAP-kinase family. Phosphatases are further discussed in Chapter 18, “Protein Phosphatases.” Secondly, there are the negative feedback pathways such as phosphorylation of the guanine nucleotide exchange factor SOS1 by ERK1/2 which reduces its affinity for GRB2, so suppressing the Ras signaling pathway (Buday et al., 1995; Corbalan-Garcia et al., 1996). Downstream of ERK1/2, RSK can also phosphorylate SOS1 (but not SOS2, which lacks several S/T phosphorylation sites) (Hauge and Frodin, 2006). Among the activities set in train by activation of the EGF and PDGF receptors is the generation of diacylglycerol and IP_3 by PLGG. The diacylglycerol remains in the membrane, there to act as a stimulus for protein kinase Cα (PRKCA). One of the first substrates of protein kinase Cα is the EGF receptor itself. The kinase activity is inhibited through phosphorylation of threonine-678, adjacent to the LRRLL sequence (return to Figure 10-6) (Hunter et al., 1984).

A third mechanism of negative feedback is the removal of cell surface receptors by endocytosis. In the case of EGF, endocytosis following low level stimulation actually induces a burst of signaling before suppressing it, due to a contribution from the internalized receptor (Schoeberl et al., 2002). Signaling only terminates when the receptors are degraded by acid hydrolases in the late endosomes. Phosphorylated receptors are preferentially endocytosed and directed by vesicular transport to the late endosomal compartment through the actions of GRB2 and CBL (an E3-ubiquitin ligase) (Figure 10-26). Both bind to receptors through their

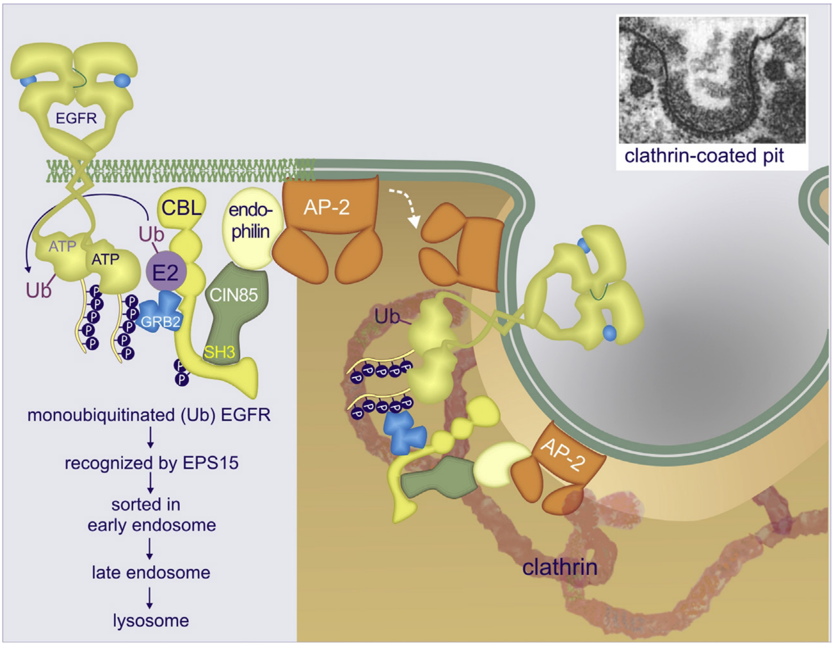


FIGURE 10-26 Removal of the EGFR from the cell surface and sorting into the lysosome pathway. Activated EGF receptors are recognized by CBL which binds either directly through a phosphotyrosine-binding motif or by interaction with the SH3 domain of GRB2. Ubiquitylation by CBL acts as a sorting signal directing the receptor into the lysosomal pathway for degradation. The receptor–Cbl complex is recognized by CIN85 and endophilin which couple the receptor to a group of proteins that includes the endocytic adaptor AP-2. Clathrin monomers are then recruited and the active EGFRs accumulate in clathrin-coated membrane pits which pinch off from the plasma membrane as endocytic vesicles. Within the intracellular network of vesicular transport pathways, the receptors are taken through the early and late endosomes toward the lysosome and destroyed.

(atypical) SH2 domains and both contribute to the clustering of receptors and their uptake into clathrin-coated vesicles (Wang and Moran, 1996; Citri and Yarden, 2006). CBL may also act as a handle, to prevent recycling of the activated EGFR to the plasma membrane (Figure 10-26).

CBL AND MONO-UBIQUITYLATION

Ubiquitylation is controlled by ubiquitin activating (E1), conjugating (E2), and ligase (E3) enzymes. It results in the attachment of the small ubiquitin protein (76 residues) to a substrate protein (for more detail see "essay: ubiquitylation and SUMOylation," Chapter 13, "Activation of the Innate Immune System"). Ubiquitins and ubiquitin-like peptides are now recognized as general signaling devices having several rolesS[5822]. Whereas attachment of four or more ubiquitins, poly-ubiquitylation, is a recognition signal for destruction by the proteasome, mono-ubiquitylation is a signal for endocytosis and in histone regulation. In the endosome, ubiquitin serves as a molecular signature on trafficking cargos. CBL, an E3 ligase, catalyses ubiquitylation of the EGFR so that it is recognized by proteins bearing ubiquitin-binding domains. For instance the adaptor protein EPS15 has two ubiquitin-interacting motifs that interact with the "protein-sorting" machinery.

A FAMILY OF MAP-KINASE-RELATED PROTEINS

When ERK1 and -2 were cloned, it became apparent that they are members of a substantial family, the MAP kinases. Based on sequence analysis and the composition of their activation segments, they may be classified into three groups, each operating in more-or-less different signal transduction pathways (Figure 10-27).

ERK

- MAPK3 (ERK1) and MAPK1 (ERK2). These are the "prototypic" or "classical" MAP kinases, operating mainly in mitogen-activated signal transduction pathways as described here.
- MAPK6 (ERK3) and MAPK4 (ERK4). These are distinguished from other ERKs by the absence of a tyrosine in the activation segment. Thus they possess only a single (serine) phosphorylation site in the motif –SEG-. Little is known about their upstream regulators and substrates but they have been shown to form complexes with MAPKAPK5 leading to mutual activation (Kant et al., 2006).
- MAPK7 (ERK5). Originally called Big MAP kinase or BMK1 because of its C-terminal domain that gives rise to a protein twice the size of ERK1/2.

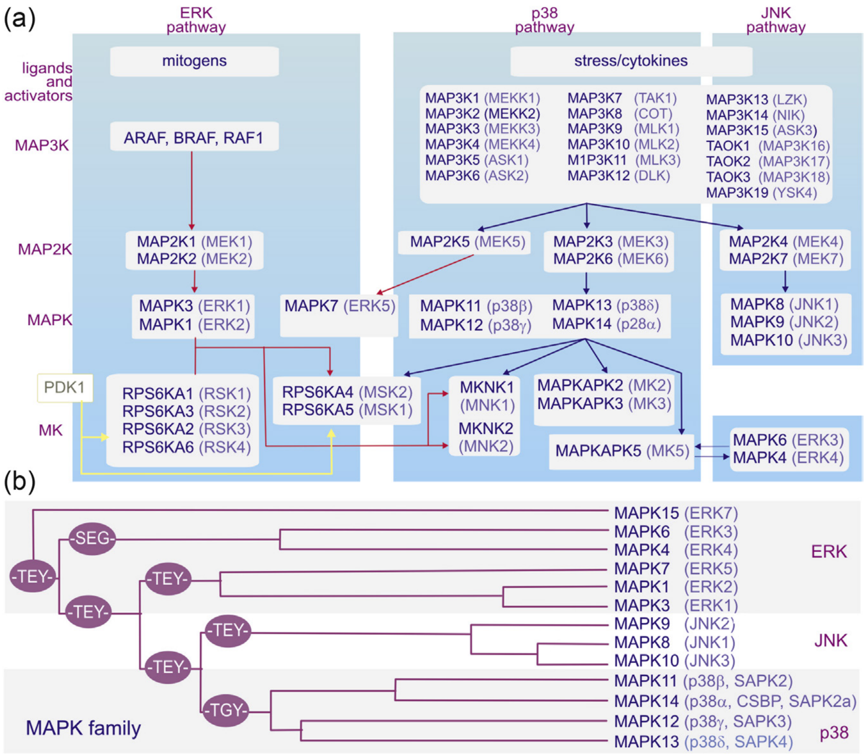


FIGURE 10-27 Parallel MAP-kinase pathways. (a) The MAP kinase cascades occur in three general pathways. One emanating from growth factor receptors and leading to the activation of the ERK kinases, one emanating from stress and cytokines and leading to activation of p38 kinases, and one emanating from stress and cytokines and resulting in the activation of JNK kinases. The ERK and p38 cascades continue with the activation of MAP-kinase-activated kinases (MK), an event that may require support from the protein kinase PDK1. The JNK pathway lacks downstream kinases. Cross talk occurs between the pathways. MAPK6 and -7 stand apart, they interact with MAPKAPK5 leading to mutual phosphorylation and activation. MAPK15 (ERK7) is not in the scheme. (b) The MAPK family (this time referring uniquely to the third kinase in the three-tier cascades) is classified into three groups based on sequence homology. They are further characterized by the phosphorylation motif of their activation segment (SEG, TEY, TGY, or TPY). MAPK15 (ERK7) stands apart.

It is activated in a complex composed of MAP2K2 and MAP3K2, kinases that normally operate in the "p38 pathway." It is essential for growth factor-induced activation of cell cycle progression and is involved in the regulation of TP53 by disrupting the PML–MDM2 interaction (Yang et al., 2013).

- MAPK15 (ERK7). The protein is quite different from all the rest and it stands out only by the lack of understanding about its activation pathway and functions.

p38

- The p38/HOG family of protein kinases. These came to light in genetic deletion studies in yeast (*S. cerevisiae*), as a kinase involved in the generation of glycerol in response to osmotic stress (Brewster et al., 1993). HOG1 induces expression of glycerol-3-phosphate dehydrogenase, related to glycerol synthesis (Albertyn et al., 1994). In mammalian cells, it is also activated in response to stress stimuli and that is why they were initially referred to as Stress-Activated Protein Kinase or SAPK. The subfamily of p38 kinases comprises four members MAPK11 (p38β), MAPK12 (p38α), MAPK13 (p38γ), and MAPK14 (p38α) (Chen et al., 2001).

JNK

- Discovered as JUN N-terminal phosphorylating and UV-activated kinase (Pulverer et al., 1991; Hibi et al., 1993; Kyriakis and Avruch, 2001). Members of the JNK subfamily also emerged in experiments in which rat livers were challenged by injection of cycloheximide, an inhibitor of protein synthesis used as a cell "stressant" (Kyriakis and Avruch, 1990). Other activating factors include growth factor deprivation, irradiation, or treatment with inflammatory cytokines (IL1β, TNF).

Cycloheximide, a product of the bacterium *Streptomyces griseus,* is an inhibitor of protein biosynthesis in eukaryotic organisms. It interferes with the activity of peptidyl transferase activity, thus blocking translational elongation.

Each of the pathways, shown in Figure 10-27 involves a kinase cascade, comprising a MAP3K and MAP2K and culminating in the phosphorylation and activation of the particular MAPK. Each contains a dual phosphorylation site, TEY, TPY, or TGY. ERKs 3 and 4 are an exception. These parallel pathways of activation may operate individually or in combination to initiate specific patterns of gene expression. Although it is generally accepted that the ERK pathway responds to mitogen stimulation, and the JNK and p38 pathways to stress and inflammation, cross-talk undoubtedly occurs. We elaborate further on the roles of JNK and p38 and their role in the regulation of the innate immune response (inflammation) in Chapter 13, "Activation of the Innate Immune System."

MAP KINASES IN OTHER ORGANISMS

Pathways regulated by MAP kinases are present in all eukaryotic organisms (Whitmarsh and Davis, 1998). In yeast (*S. cerevisiae*), processes regulated by MAP kinases include mating, sporulation, maintenance of cell wall integrity, invasive growth, pseudohyphal growth, and osmoregulation. MAP kinase is a regulator of embryonic development and the immune response in *Drosophila*. It acts as a regulator in slime molds, plants, and fungi.

OTHER BRANCHES OF THE EGFR SIGNALING PATHWAYS

The Ca^{2+}/calmodulin pathway

The elevation of cytosol Ca^{2+} following activation of PLCγ (PKCG) results in widespread protein phosphorylation by serine/threonine kinases. These include the broad spectrum Ca^{2+}-calmodulin-dependent protein kinase II (CAMK2), myosin light chain kinase (MYLK), phosphorylase kinase (PHKG1), and EF-2 kinase (EEF2K). All of these are activated by the Ca^{2+}/calmodulin complex. The level of phosphorylation of a particular substrate at any time must be determined by the rates of both phosphorylation and dephosphorylation. Ca^{2+}/calmodulin can affect this balance through activation of the protein phosphatase calcineurin (PPP3CA). This leads to the activation of transcription factors that play essential roles in the activation of T-lymphocytes. Clearly, Ca^{2+} is an extremely versatile second messenger modulating numerous intracellular signals. Ca^{2+} effectors are extensively discussed in Chapter 6, “Intracellular Calcium.” We return to Ca^{2+} and calcineurin in T-lymphocytes in Chapter 15, “Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases.”

Activation of PI3-kinase

Association of the adaptor subunit of PI3-kinase (PIK3R1 or p85) with the tyrosine-phosphorylated receptor positions the attached p110 catalytic subunit (PIK3CA), at the membrane where it phosphorylates inositol phospholipids at the third position of the inositol ring (return to Figure 10-9 for domain architecture of PIK3R1). The p110 catalytic subunit can also be activated directly by RAS. This results in activation of protein kinase B (AKT), a kinase of prime importance in cell survival, proliferation, motility, and glucose metabolism. This pathway is considered in Chapter 16, “Signaling through the Insulin Receptor.”

Direct phosphorylation of STAT transcription factors

To provide evidence that growth factors can also initiate relatively short signaling events we show the example of activation of the STAT transcription factors. Indeed, the simplest way by which plasma-membrane receptors alter gene expression is through the direct phosphorylation of transcription factors. The STATs came to light as targets of interferon receptors (inflammation control) but they also mediate EGF and PDGF signaling (Vignais et al., 1996; Zhong et al., 1994). STAT transcription factors possess an SH2 domain that enables them to associate with the phosphotyrosines of activated receptors (Figure 10-28). They themselves are then phosphorylated on tyrosine residues, causing them to form dimers (example STAT1/3) that translocate to the nucleus where they promote transcription of early response genes such as FOS. The STAT dimer formed after phosphorylation by the PDGF receptor was originally described as Sis-inducible factor (SIF), because it was observed in cells exposed to the

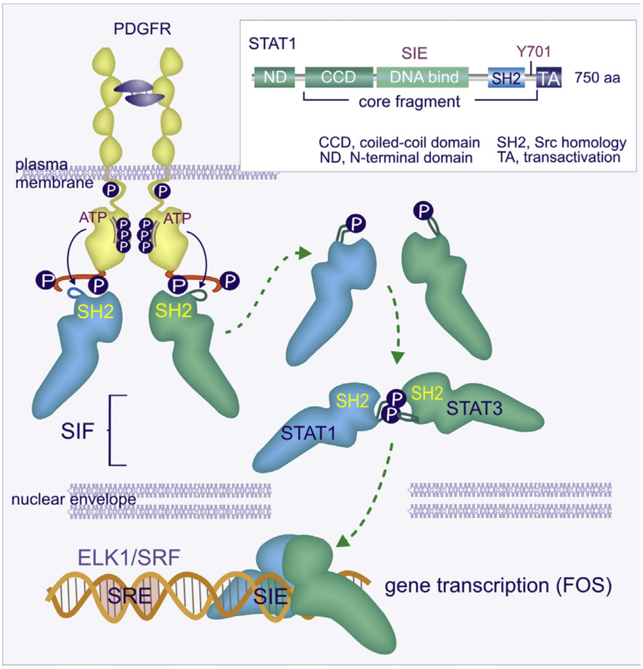


FIGURE 10-28 Direct phosphorylation of STAT transcription factors by the PDGFR. STAT1 and STAT3 bind to the tyrosine-phosphorylated platelet-derived growth factor receptor (PDGFR) and themselves become phosphorylated. They form a dimer (in this context also known as the Sis-inducible factor or SIF) which translocates to the nucleus, where it binds to a Sis-inducible element (SIE) within the FOS promoter. A similar scenario has been described for the EGFR.

viral oncogene product v-Sis. This is closely related to PDGF and activates the same signal transduction pathway (Wagner et al., 1990; Doolittle et al., 1983). We return to STAT transcription factors in Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases."

Pathway switching by transactivation

In Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," we have shown that β-arrestin, a MAPK kinase scaffold protein, binds the β-adrenoceptor and switches signaling events from a classic G protein-coupled signal, involving adenylyl cyclase, into a MAPK signal. This phenomenon is named "pathway switching" (return to Chapter 3, Section "Arresting the β-adrenoreceptor signal: pathway switching and the role of G-protein receptor kinase and arresting"). Another way of switching is by receptor transactivation (Daub et al., 1997; Prenzel et al., 1999). As an example, activation of muscarinic acetylcholine or thrombin receptors releases HB-EGF (an EGF-like factor) by cleavage from its inactive membrane-bound precursor by the metalloprotease ADAM17 (Reiss and Saftig, 2009). HB-EGF then acts in an autocrine/paracrine manner to stimulate the EGF receptor, resulting in the activation of ERK (Figure 10-29) (Prenzel et al., 1999; Zwick et al., 1997). In effect in the process of transactivation, the first ligand induces the release of a second, quite unrelated

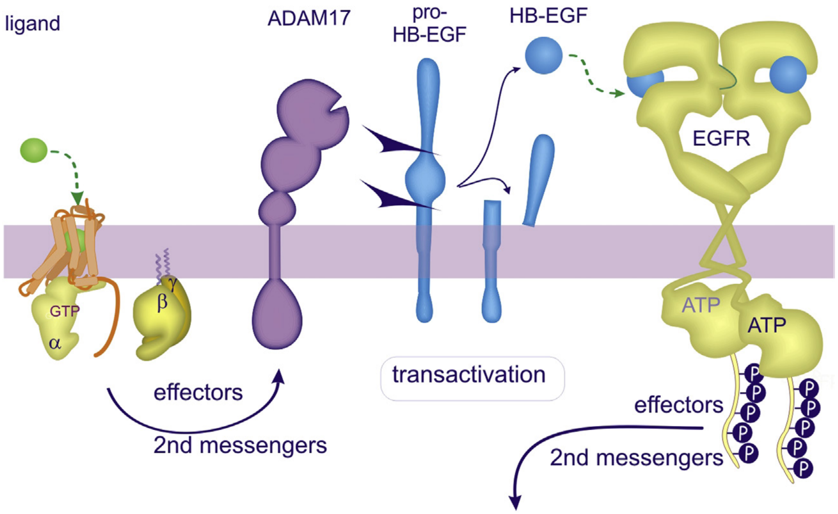


FIGURE 10-29 Transactivation of receptors. Activation of the 7TM receptor results in the production of second messengers which in turn through activation of effectors cause the activation of ADAM17, a transmembrane endoproteinase. It cleaves the HB-EGF from its membrane-bound precursor which then binds to the EGF receptor and induces yet another set of intracellular signaling pathways that include the Ras-ERK kinase pathway.

ligand, which in turn sets in train its own distinct signaling pathways. Receptor transactivation expands enormously the repertoire of signaling systems that a cell can apply in mounting a response. In this example, the mechanism of transactivation depends, in the first place, upon intracellular signals that activate the transmembrane metalloproteinase that has its catalytic site situated in the extracellular domain. It is still not clear how ADAM17 is activated. In some cells it requires a cytoplasmic domain and responds to phosphorylation by protein kinase C, (Prakasam et al., 2014) in others it does not.

Pathway switching, transactivation, and metastatic progression of colorectal cancer

Both pathway switching and transactivation make contributions in the malignant behavior of already transformed epithelial cells of the intestinal tract. The inflammatory conditions caused by tumor cells raise the local production of prostaglandins by leukocytes, in particular prostaglandin E2 (PGE2) which acts through G protein-coupled receptors (PTGER). Of the four receptors characterized, PTGER2 and -4 were shown to cause transactivation of EGF receptors as described in the previous paragraph (Pai et al., 2002; Regan, 2003). In addition, PTGER2 and -4 bind to β-arrestin to activate SRC. This then, both through transactivation and pathway switching, contributes to cell migration in vitro and metastasis in vivo (Buchanan et al., 2006). Perhaps aspirin, that old favorite among remedies, might offer some protection against colorectal tumor invasion through its action as an inhibitor of prostaglandin production (Wang and DuBois, 2006).

References

Arkhipov, A., Shan, Y., Das, R., Endres, N.F., Eastwood, M.P., Wellerr, D.E., Kuriyan, J., Shaw, D.E., 2013. Architecture and membrane interactions of the EGF receptor. Cell 152, 557–569.

Anderson, R.B.D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F., Pawson, T., 1990. Binding of SH2 domains of phospholipase Cg_1, GAP, and Src to activated growth factor receptors. Science 250, 979–982.

Aronheim, A., Engelberg, D., Li, N., al-Alawi, N., Schlessinger, J., Karin, M., 1994. Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. Cell 78, 949–961.

Ahn, N.G., Weiel, J.E., Chan, C.P., Krebs, E.G., 1990. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. J. Biol. Chem. 265, 11487–11494.

Adachi, M., Fukuda, M., Nishida, E., 1999. Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. EMBO J. 18, 5347–5358.

Adachi, M., Fukuda, M., Nishida, E., 2000. Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism. J. Cell Biol. 148, 849–856.

Aplin, A.E., Stewart, S.A., Assoian, R.K., Juliano, R.L., 2001. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. 153, 273–282.

Albertyn, J., Hohmann, S., Thevelein, J.M., Prior, B.A., 1994. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell Biol. 14, 4135–4144.

Bazley, L.A., Gullick, W.J., 2005. The epidermal growth factor receptor family. Endocr. Relat. Cancer 12, S17–S27.

Burg, D.L., Furlong, M.T., Harrison, M.L., Geahlen, R.L., 1994. Interactions of Lyn with the antigen receptor during B cell activation. J. Biol. Chem. 269, 28136–28142.

Brewer, M.R., Choi, S.H., Alvarado, D., Moravceci, K., Pozzi, A., Lemmon, A., Carpenter, G., 2009. The juxtamembrane region off the EGF receptor functions as an activating domain. Mol. Cell 34, 641–651.

Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., Margolis, B., 1994. A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. J. Biol. Chem. 269, 32031–32034.

Burgering, B.M., Freed, E., van der Voorn, L., McCormick, F., Bos, J.L., 1994. Platelet-derived growth factor-induced p21ras-mediated signaling is independent of platelet-derived growth factor receptor interaction with GTPase-activating protein or phosphatidylinositol-3-kinase. Cell Growth Differ. 5, 341–347.

Bolcome, R.E., Chan, J., 2010. Constitutive MEK1 activation rescues anthrax lethal toxin-induced vascular effects in vivo. Infect. Immun. 78, 5043–5053.

Baines, A.T., Xu, D., Der, C.J., 2011. Inhibition of Ras for cancer treatment: the search continues. Future Med. Chem. 3, 1787–1808.

Brunet, A., Pouyssegur, J., 1996. Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science 272, 1652–1655.

Barber, M.F., Michishita-Kioi, E., Xi, Y., Tasselli, L., Kioi, M., Moqtaderi, Z., Tennen, R.I., Paredes, S., Young, N.L., Chen, K., Struhl, K., Garcia, B.A., Gozani, O., Li, W., Chua, K.F., 2012. SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. Nature 487, 114–118.

Bakiri, L., Lallemand, D., Bossy-Wetzel, E., Yaniv, M., 2000. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. EMBO J. 19, 2056–2068.

Bhattacharyya, R.P., Remenyi, A., Good, M.C., Bashor, C.J., Falick, A.M., Lim, W.A., 2006. The Ste5 scaffold allosterically modulates signaling output of the yeast mating pathway. Science 311, 822–826.

Bashor, C.J., Helman, N.C., Yan, S., Lim, W.A., 2008. Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics. Science 319, 1539–1543.

Buday, L., Warne, P.H., Downward, J., 1995. Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos. Oncogene 11, 1327–1331.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., Gustin, M.C., 1993. An osmosensing signal transduction pathway in yeast. Science 259, 1760–1763.

Buchanan, F.G., Gorden, D.L., Matta, P., Shi, Q., Matrisian, L.M., DuBois, R.N., 2006. Role of beta-arrestin 1 in the metastatic progression of colorectal cancer. Proc. Natl. Acad. Sci. U.S.A. 103, 1492–1497.

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D., Kuriyan, J., 1998. The structural basis of the activation of Ras by Sos. Nature 394, 337–343.

Brennan, D.F., Dar, A.C., Hertz, N.T., Chao, W.C., Burlingame, A.L., Shokat, K.M., Barford, D., 2011. A Raf-induced allosteric transition of KSR stimulates phosphorylation of MEK. Nature 472, 366–369.

Collett, M.S., Erkison, R.L., 1978. Protein kinase activity associated with the avaion sarcoma virus src gene product. PNAS U.S.A. 75, 2021–2024.

Cantrell, D.A., 1996. T cell antigen receptor signal transduction pathways. Cancer Surv. 27, 165–175.

Carpenter, G., King, L., Cohen, S., 1979. Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. Nature 276, 409–410.

Cho, H.S., Mason, K., Ramyar, K.X., Stanley, A.M., Gabelli, S.B., Denney, D.W., Leahy, D.J., 2003. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature 421, 756–760.

Citri, A., Yarden, Y., 2006. EGF-ERBB signalling: towards the systems level. Nat. Rev. Mol. Cell Biol. 7, 505–516.

Carter, R.E., Sorkin, A., 2006. Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera. J. Biol. Chem. 273, 35000–35007.

Chang, E.H., Furth, M.E., Scolnick, E.M., Lowy, D.R., 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature 297, 479–483.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., Cobb, M.H., 2001. MAP kinases. Chem. Rev. 101, 2449–2476.

Choi, K.Y., Satterberg, B., Lyons, D.M., Elion, E.A., 1994. Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell 78, 499–512.

Corbit, K.C., Trakul, N., Eves, E.M., Diaz, B., Marshall, M., Rosner, M.R., 2003. Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J. Biol. Chem. 278, 13061–13068.

Corbalan-Garcia, S., Yang, S.S., Degenhardt, K.R., Bar-Sagi, D., 1996. Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2. Mol. Cell Biol. 16, 5674–5682.

Citri, A., Skaria, K.B., Yarden, Y., 2003. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp. Cell Res. 284, 54–65.

Defize, L.H., Moolenaar, W.H., van der Saag, P.T., de Laat, S.W., 1986. Dissociation of cellular responses to epidermal growth factor using anti-receptor monoclonal antibodies. EMBO J. 5, 1187–1192.

Dumaz, N., Light, Y., Marais, R., 2002. Cyclic AMP blocks cell growth through Raf-1-dependent and Raf-1-independent mechanisms. Mol. Cell Biol. 22, 3717–3728.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D., Vande Woude, G.F., 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280, 734–737.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B.A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G.J., Bigner, D.D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J.W., Leung, S.Y., Yuen, S.T., Weber, B.L., Seigler, H.F., Darrow, T.L., Paterson, H., Marais, R., Marshall, C.J., Wooster, R., Stratton, M.R., Futreal, P.A., 2002. Mutations of the BRAF gene in human cancer. Nature 417, 949–954.

Dimitri, C.A., Dowdle, W., MacKeigan, J.P., Blenis, J., Murphy, L.O., 2005. Spatially separate docking sites on ERK2 regulate distinct signaling events in vivo. Curr. Biol. 15, 1319–1324.

Dobrikov, M.I., Dobrikova, E.Y., Gromeier, M., 2013. Dynamic regulation of the translation initiation helicase complex by mitogenic signal transduction to eukaryotic translation initiation factor 4G. Mol. Cell Biol. 33, 937–946.

Dalton, S., Treisman, R., 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell 68, 597–612.

Drobic, B., Perez-Cadahia, B., Yu, J., Kung, S.K., Davie, J.R., 2010. Promoter chromatin remodelling of immediate-early genes is mediated through H3 phosphorylation at either serein 28 or 10 by the MSK1 multi-protein complex. Nucleic Acids Res. 38, 3196–3208.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., Antoniades, H.N., 1983. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275–277.

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., Ullrich, A., 1997. Signal characteristics of G protein-transactivated EGF receptor. EMBO J. 16, 7032–7044.

Eckhart, W., Hutchinson, M.A., Hunter, T., 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell 18, 925–933.

Endres, N.F., Das, R., Smith, A.W., Arkhipv, A., Kovacs, E., Huang, Y., Pelton, J.G., Shan, Y., Shaw, D.E., Wemm, D.E., Groves, J.T., 2013. Conformational coupling across the plasma membrane in activation off the EGF receptor. Cell 152, 543–556.

Escobedo, J.A., Navankasattusas, S., Kavanaugh, W.M., Milfay, D., Fried, V.A., Williams, L.T., 1991. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF b-receptor. Cell 65, 75–82.

Elion, E.A., 2001. The Ste5p scaffold. J. Cell Sci. 114, 3967–3978.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E.L.L., Tate, J., Punta, M., 2014. Pfam: the protein families database. Nucleic Acids Res. 42, D222–D230 Database Issue.

Feramisco, J.R., Gross, M., Kamata, T., Rosenberg, M., Sweet, R.W., 1984. Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells. Cell 38, 109–117.

Feramisco, J.R., Clark, R., Wong, G., Arnheim, N., Milley, R., McCormick, F., 1985. Transient reversion of ras oncogene-induced cell transformation by antibodies specific for amino acid 12 of ras protein. Nature 314, 639–642.

Fortini, M.E., Simon, M.A., Rubin, G.M., 1992. Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. Nature 355, 559–561.

Grangeasse, C., Doublet, P., Vaganay, E., Vincent, C., Deleage, G., Duclos, B., Cozzone, A.J., 1997. Characterization of a bacterial gene encoding an autophosphorylating protein tyrosine kinase. Gene 204, 259–265.

Giancotti, F.G., 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. 9, 691–700.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A., Carraway 3rd, K.L., 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. U.S.A. 91, 8132–8136.

Gerrett, T.P., McKern, N.M., Lou, M., et al., 2002. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor-α. Cell 110, 763–773.

Gilligan, A., Prentki, M., Knowles, B.B., 1990. EGF receptor down-regulation attenuates ligand-induced second messenger formation. Exp. Cell Res. 187, 134–142.

Gonzalez, F.A., Gross, D.J., Heppel, L.A., Webb, W.W., 1988. Studies on the increase in cytosolic free calcium induced by epidermal growth factor, serum, and nucleotides in individual A431 cells. J. Cell Physiol. 135, 269–276.

van der Geer, P., Pawson, T., 1995. The PTB domain: a new protein module implicated in signal transduction. Trends Biochem. Sci. 20, 277–280.

Gale, N.W., Kaplan, S., Lowenstein, E.J., Schlessinger, J., Bar-Sagi, D., 1993. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature 363, 88–92.

Gureasko, J., Galush, W.J., Boykevisch, S., Sondermann, H., Bar-Sagi, D., Groves, J.T., Kuriyan, J., 2008. Membrane-dependent signal integration by the Ras activator Son of sevenless. Nat. Struc. Mol. Biol. 15, 452–461.

Gureasko, J., Kuchment, O., Lakina, D.L., Sondermann, H., Bar-Sagi, D., Kuriyan, J., 2010. Role of the histone domain in the autoinhibition ad activation of the Ras activator Son of sevenless. PNAS U.S.A. 107, 3430–3435.

Galanis, A., Yang, S.H., Sharrocks, A.D., 2001. Selective targeting of MAPKs to the ETS domain transcription factor SAP-1. J. Biol. Chem. 276, 965–973.

Good, M., Tang, G., Singleton, J., Remenyi, A., Lim, W.A., 2009. The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation. Cell 136, 1085–1097.

Good, M.C., Zalatan, J.G., Lim, W.A., 2011. Scaffold proteins: hubs for controlling the flow of cellular information. Science 332, 680–686.

Hendriks, B.S., Orr, G., Wells, A., Wiley, H.S., Lauffenburger, D.A., 2005. Parsing ERK activation reveals quantitatively equivalent contributions from epidermal growth factor receptor and HER2 in human mammary epithelial cells. J. Biol. Chem. 280, 6157–6169.

Higaki, M., Sakaue, H., Ogawa, W., Kasuga, M., Shimokado, K., 1996. Phosphatidylinositol 3-kinase-independent signal transduction pathway for platelet-derived growth factor-induced chemotaxis. J. Biol. Chem. 271, 29342–29346.

Harris, W.A., Stark, W.S., Walker, J.A., 1976. Genetic dissection of the compound eye of *Drosophila melanogaster*. J. Physiol. 256, 415–439.

Hart, A.C., Kramer, H., Van Vactor, D.L., Paidhungat, M., Zipursky, S.L., 1990. Induction of cell fate in the *Drosophila* retina: the bride of sevenless protein is predicted to contain a large extracellular domain and seven transmembrane segments. Genes Dev. 4, 1835–1847.

Hafen, E., Basler, K., Edstroem, J.E., Rubin, G.M., 1987. Sevenless, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. Science 236, 55–63.

Hahn, C.G., Wang, H.Y., Cho, D.S., Talbot, K., Gur, R.E., Berrettini, W.H., Bakshi, K., Kamins, J., Borgmann-Winter, K.E., Siegel, S.J., Gallop, R.J., Arnold, S.E., 2006. Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. Nat. Med. 12, 824–828.

Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K., Wong, A.J., 1996. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. Nature 379, 560–564.

Hekman, M., Wiese, S., Metz, R.S., Albert, S., Troppmair, J., Nickel, J., Sendtner, M., Rapp, U.R., 2004. Dynamic changes in C-Raf phosphorylation and 14-3-3 protein binding in response to growth factor stimulation: differential roles of 14-3-3 protein binding sites. J. Biol. Chem. 279, 14074–14086.

Haling, J.R., Sudhasu, J., Yen, I., Sideris, S., Sanndoval, W., Phung, W., Bravo, B.J., Giannetti, A.M., Peck, Q., Masselot, A., Morales, T., Smith, D., Brandhuber, B.K., Hymowitz, S.G., Malek, S., 2014. Structure of the BRAF-MEK complex reveals a kinase-activity independent role for BRAF in MAPK signaling. Cancer Cell 26, 402–413.

Heidorn, S.J., Milagre, C., Whittakers, S., Nourry, A., Niculescu-Duvas, I., Dhomen, H., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C., Marais, R., 2010. Kinase-dead BRAF and congenic RAS cooperate to drive tumor progression through CRAF. Cell 140, 209–221.

Herber, B., Truss, M., Beato, M., Muller, R., 1994. Inducible regulatory elements in the human cyclin D1 promoter. Oncogene 9, 2105–2107.

Hauge, C., Frodin, M., 2006. RSK and MSK in MAP kinase signalling. J. Cell Sci. 119, 3021–3023.

Hunter, T., Ling, N., Cooper, J.A., 1984. Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311, 480–483.

Hibi, M., Lin, A., Smeal, T., Minden, A., Karin, M., 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. 7, 2135–2148.

Jura, N., Endres, F.N., Engel, K., Deindl, S., Das, R., Lamers, M.H., Wemmer, D.E., Zhang, X., Kuriyan, J., 2009. Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. Cell 137, 1293–1307.

Jones, R.B., Gordus, A., Krall, J.A., MacBeath, G., 2006. A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 439, 168–174.

Jones, S., Vignais, M.-L., Broach, J.R., 1991. The CDC25 protein of *S. cerevisiae* promotes exchange of guanine nucleotides bound to ras. Mol. Cell Biol. 11, 2641–2646.

Jansen, H.W., Ruckert, B., Lurz, R., Bister, K., 1983. Two unrelated cell-derived sequences in the genome of avian leukemia and carcinoma inducing retrovirus MH2. EMBO J. 2, 1969–1975.

Kirken, R.A., Rui, H., Evans, G.A., Farrar, W.L., 1993. Characterization of an interleukin-2 (IL-2)-induced tyrosine phosphorylated 116-kDa protein associated with the IL-2 receptor β-subunit. J. Biol. Chem. 268, 22765–22770.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., Pawson, T., 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252, 668–674.

Kavanaugh, W.M., Williams, L.T., 1994. An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science 266, 1862–1865.

Kramer, H., Cagan, R.L., Zipursky, S.L., 1991. Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. Nature 352, 207–212.

Kornfeld, K., 1997. Vulval development in *Caenorhabditis elegans*. Trends Genet. 13, 55–61.

Koide, H., Satoh, T., Nakafuku, M., Kaziro, Y., 1993. GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 90, 8683–8686.

Kolch, W., 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J. 351 (Pt 2), 289–305.

Kallunki, T., Su, B., Tsigelny, I., Sluss, H.K., Derijard, B., Moore, G., Davis, R., Karin, M., 1994. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev. 8, 2996–3007.

Kallunki, T., Deng, T., Hibi, M., Karin, M., 1996. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 87, 929–939.

Knauf, U., Tschopp, C., Gram, H., 2001. Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. Mol. Cell Biol. 21, 5500–5511.

Kruijer, W., Cooper, J.A., Hunter, T., Verma, I.M., 1984. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature 312, 711–716.

Kaikkonen, S., Makkonen, H., Rytinki, M., Palvimo, J.J., 2010. SUMOylation can regulate the activity of ETS-like transcription factor 4. Biochim. Biophys. Acta 1799, 555–560.

Kahan, C., Seuwen, K., Meloche, S., Pouyssegur, J., 1992. Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. Evidence for thrombin-induced signals different from phosphoinositide turnover and adenylylcyclase inhibition. J. Biol. Chem. 267, 13369–13375.

Kornfeld, K., Hom, D.B., Horvitz, H.R., 1995. The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. Cell 83, 903–913.

Kolch, W., 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat. Rev. Mol. Cell Biol. 6, 827–837.

Kant, S., Schumacher, S., Singh, M.K., Kispert, A., Kotlyarov, A., Gaestel, M., 2006. Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK. J. Biol. Chem. 281, 35511–35519.

Kyriakis, J.M., Avruch, J., 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81, 807–869.

Kyriakis, J.M., Avruch, J., 1990. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. J. Biol. Chem. 265, 17355–17363.

Li, W., Deanin, G.G., Margolis, B., Schlessinger, J., Oliver, J.M., 1992. Fcε R1-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor bg$_2$ complex, in RBL-2H3 rat basophilic leukemia cells. Mol. Cell Biol. 12, 3176–3182.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., Schlessinger, J., 1993. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 363, 85–88.

Luetteke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O., Lee, D.C., 1993. TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell 73, 263–278.

Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A., Lee, D.C., 1999. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development 126, 2739–2750.

Liu, B.A., Jablonowoski, K., Shah, E.E., Engelman, B.W., Jones, R.B., Nash, P.D., 2010. SH2 domains recognize contextual peptide sequence information to determine selectivity. Mol. Cell Proteomics 9, 2391–2404.

Liu, X.Q., Pawson, T., 1991. The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. Mol. Cell Biol. 11, 2511–2516.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D., Schlessinger, J., 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 70, 431–442.

Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., Stehelin, D., 1983. A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. Nature 306, 395–397.

Ladha, M.H., Lee, K.Y., Upton, T.M., Reed, M.F., Ewen, M.E., 1998. Regulation of exit from quiescence by p27 and cyclin D1-CDK4. Mol. Cell Biol. 18, 6605–6615.

Lee, W.H., Shew, J.Y., Hong, F.D., Sery, T.W., Young, L.J., Bookstein, R., Lee, E.Y., 1987. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature 329, 642–645.

Lamson, R.E., Takahashi, S., Winters, M.J., Pryciak, P.M., 2006. Dual role for membrane localization in yeast MAP kinase cascade activation and its contribution to signaling fidelity. Curr. Biol. 16, 618–623.

Lozano, J., Xing, R., Cai, Z., Jensen, H.L., Trempus, C., Mark, W., Cannon, R., Kolesnick, R., 2003. Deficiency of kinase suppressor of Ras1 prevents oncogenic ras signaling in mice. Cancer Res. 63, 4232–4238.

Luttrell, L.M., Lefkowitz, R.J., 2002. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J. Cell Sci. 115455–115465.

Manning, G., Young, S.L., Miller, W.T., Zha, Y., 2008. The protist, *Monosiga brevicollis*, has tyrosine kinase signaling network more elaborate and diverse than found in known metazoan. PNAS U.S.A. 105, 9674–9679.

Miettine, P.J., Berger, J.E., Meneses, J., Phung, Y., Pedersen, R.A., Werb, Z., Derynck, R., 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 37, 337–341.

Mi, L.-Z., Lu, C., Li, Z., Nishida, N., Walz, T., Springer, T.A., 2011. Simultaneous visualization of the extracellular and cytoplasmic domains of the epidermal growth factor receptor. Nat. Struct. Mol. Biol. 18, 984–989.

Medema, R.H., de Vries Smits, A.M., van der Zon, G.C., Maassen, J.A., Bos, J.L., 1993. Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21ras. Mol. Cell Biol. 13, 155–162.

Muroya, K., Hattori, S., Nakamura, S., 1992. Nerve growth factor induces rapid accumulation of the GTP-bound form of p21ras in rat pheochromocytoma PC12 cells. Oncogene 7, 277–281.

Meisenhelder, J., Suh, P.G., Rhee, S.G., Hunter, T., 1989. Phospholipase Cγ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell 57, 1109–1122.

Matsuda, M., Mayer, B.J., Fukui, Y., Hanafusa, H., 1990. Binding of transforming protein, P47gag-crk, to a broad range of phosphotyrosine-containing proteins. Science 248, 1537–1539.

Moran, M.F., Koch, C.A., Anderson, D., Ellis, C., England, L., Martin, G.S., Pawson, T., 1990. Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc. Natl. Acad. Sci. U.S.A. 87, 8622–8626.

Margarit, S.M., Sondermann, H., Hall, B.E., Nagar, B., Hoelz, A., Pirruccello, M., Bar-Sagi, D., Kuriyan, J., 2003. Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS. Cell 112, 685–695.

Moodie, S.A., Willumsen, B.M., Weber, M.J., Wolfman, A., 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 260, 1658–1661.

Mo, Y., Ho, W., Johnston, K., Marmorstein, R., 2001. Crystal structure of a ternary SAP-1/SRF/c-fos SRE DNA complex. J. Mol. Biol. 314, 495–506.

Murphy, L.O., Smith, S., Chen, R.H., Fingar, D.C., Blenis, J., 2002. Molecular interpretation of ERK signal duration by immediate early gene products. Nat. Cell Biol. 4, 556–564.

Murphy, L.O., MacKeigan, J.P., Blenis, J., 2004. A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration. Mol. Cell Biol. 24, 144–153.

Matheny, S.A., Chen, C., Kortum, R.L., Razidlo, G.L., Lewis, R.E., White, M.A., 2004. Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. Nature 427, 256–260.

Muller, J., Ory, S., Copeland, T., Piwnica-Worms, H., Morrison, D.K., 2001. C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol. Cell 8, 983–993.

Naccache, P.H., Gilberert, C., Caon, A.C., et al., 1990. Selective inhibition off human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. Blood 76, 2098–2104.

Nguyen, A., Burack, W.R., Stock, J.L.J, Kortum, R., Chaika, O.V., Afkarian, M., Muller, W.J., Murphy, K.M., Morrison, D.K., Lewis, R.E., McNeish, J., Shaw, A.S., 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol. Cell Biol. 22, 3035–3045.

Ottensmeyer, F.P., Beniac, D.R., Luo, R.Z., Yip, C.C., 2000. Mechanism of transmembrane signaling: insulin binding and the insulin receptor. Biochemistry 39, 12103–12112.

O'Neill, T.J., Craparo, A., Gustafson, T.A., 1994. Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. Mol. Cell Biol. 14, 6433–6442.

Oliver, S.G., 1997. From gene to screen with yeast. Curr. Opin. Genet. Dev. 7, 405–409.

Ory, S., Zhou, M., Conrads, T.P., Veenstra, T.D., Morrison, D.K., 2003. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. Curr. Biol. 13, 1356–1364.

Oskarsson, T., Trumpp, A., 2005. The Myc trilogy: lord of the RNA polymerases. Nat. Cell Biol. 7, 215–217.

Pandiella, A., Beguinot, L., Velu, T.J., Meldolesi, J., 1988. Transmembrane signalling at epidermal growth factor receptors overexpressed in NIH 3T3 cells. Phosphoinositide hydrolysis, cytosolic Ca^{2+} increase and alkalinization correlate with epidermal-growth-factor-induced cell proliferation. Biochem. J. 254, 223–228.

Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjere, F., Auberger, P., Pouyssegur, J., 1999. Defective thymocyte maturation in p44 MAPK kinase (ERK1) knockout mice. Science 286, 1374–1377.

Plotnikov, A., Chuderland, D., Karamansha, Y., Livnah, O., Seger, R., 2011. Nuclear extracellular signal-regulated kinase 1 and 2 translocation is mediated by casein kinase 2 and accelerated autophosphorylation. Mol. Cell Biol. 31, 3515–3530.

Pages, G., Brunet, A., L'Allemain, G., Pouyssegur, J., 1994. Constitutive mutant and putative regulatory serine phosphorylation site of mammalian MAP kinase kinase (MEK1). EMBO J. 13, 3003–3010.

Pearce, L.R., Atanassova, N., Banton, M.C., Bottomley, B., van der Klaauw, A.A., et al., 2013. KSR2 mutations are associated with obesity, insulin resistance and impaired cellular fuel oxidation. Cell 155, 765–777.

Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E., Woodgett, J.R., 1991. Phosphorylation of c-jun mediated by MAP kinases. Nature 353, 670–674.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., Ullrich, A., 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402, 884–888.

Prakasam, H.S., Gallo, L.I., Li, H., Ruiz, W.G., Hallows, K.R., Apodaca, G., 2014. A1 adenosine receptor–stimulated exocytosis in bladder umbrella cells requires phosphorylation of ADAM17 Ser-811 and EGF receptor transactivation. Mol. Biol. Cell 25, 3798–3812.

Pai, R., Soreghan, B., Szabo, I.L., Pavelka, M., Baatar, D., Tarnawski, A.S., 2002. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat. Med. 8, 289–293.

Pierre, S., Bats, A.S., Coumoul, X., 2011. Understanding S.O.S. (son of sevenless). Biochem. Pharm. 82, 1049–1056.

Robinson, D.R., Wu, Y.M., Lin, S.F., 2000. The protein tyrosine kinase family of the human genome. Oncogene 19, 5548–5557.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., Bowtell, D., 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363, 83–85.

Ray, L.B., Sturgill, T.W., 1987. Rapid stimulation by insulin of a serine/threonine kinase in 3T3- L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc. Natl. Acad. Sci. U.S.A. 84, 1502–1506.

Rapp, U.R., Goldsborough, M.D., Mark, G.E., Bonner, T.I., Groffen, J., Reynolds Jr., F.H., Stephenson, J.R., 1983. Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. U.S.A. 80, 4218–4222.

Rajakulendran, T., Sahmi, M., Lefrançois, M., Sicheri, F., Therrien, M., 2009. Dimerization-dependent mechanism drives RAF catalytic activation. Nature 461, 542–545.

Reiss, K., Saftig, P., 2009. The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. Semin. Cell Dev. Biol. 20, 126–137.

Regan, J.W., 2003. EP2 and EP4 prostanoid receptor signaling. Life Sci. 74, 143–153.

Roux, P.P., Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. 68, 320–344.

Suga, H., Dacre, M., de Mendoza, Shalchian-Tabrizi, K., Manning, G., Ruiz-Trillo, I., 2012. Genomic survey of pre-metazoans show deep conservation of cytoplasmic tyrosine kinases and multiple radiations of receptor tyrosine kinases. Sci. Signal 5, ra35.

Schechter, A.L., Stern, D.F., Vaidyanathan, L., Decker, S.J., Drebin, J.A., Greene, M.I., Weinberg, R.A., 1984. The neu oncogene: an erb-B-related gene coding a 185,000-Mr tumour antigen. Nature 312, 513–516.

Shi, F., Telesco, S.E., Liu, Y., Radhakrishnan, R., Lemmon, M.A., 2010. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyse autophosphorylation. PNAS U.S.A. 107, 7692–7697.

Steinkamp, M.P., Low-Nam, S.T., Yang, S., Lidke, K.A., Lidke, D.S., Wilson, B.S., 2014. ErbB3 is an active tyrosine kinase capable of homo- and heterointeractions. Mol. Cell Biol. 34, 965–977.

Stein, R.A., Staros, J.V., 2000. Evolutionary analysis of the ErbB receptor and ligand families. J. Mol. Evol. 50, 397–412.

Sternberg, M.J., Gullick, W.J., 1990. Neu receptor dimerization. Nature 339, 587.

Spaargaren, M., Defize, L.H., Boonstra, J., de Laat, S.W., 1991. Antibody-induced dimerization activates the epidermal growth factor receptor tyrosine kinase. J. Biol. Chem. 266, 1733–1739.

Saito, Y.J., Haendeler, J., Hojo, K., Yamamoto, K., Berk, B.C., 2001. Receptor heterodimerization: essential mechanism for platelet-derived growth factor-induced epidermal growth factor receptor transactivation. Mol. Cell Biol. 21, 6387–6394.

Skolnik, E.Y., Margolis, B., Mohammadi, M.E., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A., Schlessinger, J., 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell 65, 83–90.

Sadowski, I., Stone, J.C., Pawson, T., 2006. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. Mol. Cell Biol. 6, 4396–4408.

Simons, P.J., Dourmashkin, R.R., Turano, A., Phillips, D.E., Chesterman, F.C., 1967. Morphological transformation of mouse embryo cells in vitro by murine sarcoma virus (Harvey). Nature 214, 897–898.

Sweet, R.W., Yokoyama, S., Kamata, T., Feramisco, J.R., Rosenberg, M., Gross, M., 1984. The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature 311, 273–275.

Satoh, T., Endo, M., Nakafuku, M., Nakamura, S., Kaziro, Y., 1990. Platelet-derived growth factor stimulates formation of active p21ras.GTP complex in Swiss mouse 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. 87, 5993–5997.

Stern, M.J., Marengere, L.E., Daly, R.J., Lowenstein, E.J., Kokel, M., Batzer, A., Olivier, P., Pawson, T., Schlessinger, J., 1993. The human GRB2 and *Drosophila* Drk genes can functionally replace the *Caenorhabditis elegans* cell signaling gene sem-5. Mol. Biol. Cell 4, 1175–1188.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M., Hancock, J.F., 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science 264, 1463–1467.

Sonenberg, N., Gingras, A.C., 1998. The mRNA 5′cap-binding protein eIF4E and control of cell growth. Curr. Opin. Cell Biol. 10, 268–275.

Suzuki, T., Tsuzuku, J., Ajima, R., Nakamura, T., Yoshida, Y., Yamamoto, T., 2002. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev. 16, 1356–1370.

Schoeberl, B., Eichler-Jonsson, C., Gilles, E.D., Muller, G., 2002. Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. Nat. Biotechnol. 20, 370–375.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269, 230–234.

Thiel, K.W., Carpenter, G., 2007. Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. PNAS U.S.A. 104, 19238–19243.

Tanoue, T., Adachi, M., Moriguchi, T., Nishida, E., 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat. Cell Biol. 2, 110–116.

de Taisne, C., Gegonne, A., Stehelin, D., Bernheim, A., Berger, R., 1984. Chromosomal localization of the human proto-oncogene c-ets. Nature 310, 581–583.

Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wassarman, D.A., Rubin, G.M., 1995. KSR, a novel protein kinase required for RAS signal transduction. Cell 83, 879–888.

Therrien, M., Michaud, N.R., Rubin, G.M., Morrison, D.K., 1996. KSR modulates signal propagation within the MAPK cascade. Genes Dev. 10, 2684–2695.

Teis, D., Wunderlich, W., Huber, L.A., 2002. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell 3, 803–814.

Ushiro, H., Cohen, S., 1980. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J. Biol. Chem. 255, 8363–8365.

Vincent, C., Doublet, P., Grangeasse, C., Vaganay, E., Cozzone, A.J., Duclos, B., 1999. Cells of *Escherichia coli* contain a protein-tyrosine kinase, Wzc, and a phosphotyrosine-protein phosphatase. Wzb. J. Bacteriol. 181, 3472–3477.

Vinciguerra, M., Vivacqua, A., Fasanella, Gallo, A., Cuooz, C., Morano, A., Maggiolini, M., Musti, A.M., 2004. Differential phosphorylation of c-Jun and JunD in response to epidermal growth factor is determined by the structure of MAPK targeting sequences. J. Biol. Chem. 279, 9634–9641.

Vicent, G.P., Ballare, C., Nacht, A.S., Clausell, j, Subtil-Rodriguez, A., Quiles, I., Jordan, A., Beato, M., 2006. Induction of progesterone target genes requires activation of ERK and Msk kinases and phosphorylation of Histone H3. Mol. Cell 24, 367–381.

Vignais, M.L., Sadowski, H.B., Watling, D., Rogers, N.C., Gilman, M., 1996. Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins. Mol. Cell Biol. 16, 1759–1769.

Witte, O.N., Dasgupta, A., Baltimore, D., 1980. Abelson murine leukaemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283, 826–831.

Williamson, P., Merida, I., Greene, W.C., Gaulton, G., 1994. The membrane proximal segment of the IL-2 receptor b-chain acidic region is essential for IL2-dependent protein tyrosine kinase activation. Leukemia 8 (Suppl. 1), S186–S189.

Warne, P.H., Viciana, P.R., Downward, J., 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 364, 352–355.

Wellbrock, C., Karasarides, M., Marais, R., 2004. The RAF proteins take centre stage. Nat. Rev. Mol. Cell Biol. 5, 875–885.

Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., Niculescu-Duvaz, D., Good, V.M., Jones, C.M., Marshall, C.J., Springer, C.J., Barford, D., Marais, R., 2004. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116, 855–867.

Witmarsh, A.J., Davis, R.J., 2000. A central control for cell growth. Nature 403, 255–256.

Wimmer, R., Baccarini, M., 2010. Partner exchange: protein-protein interactions in the Raf pathway. Trends Biochem. Sci. 35, 660–668.

Wunderlich, W., Fialka, I., Teis, D., Alpi, A., Pfeifer, A., Parton, R.G., Lottspeich, F., Huber, L.A., 2001. A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J. Cell Biol. 152, 765–776.

Wiley, H.S., Shvartsman, S.Y., Lauffenburger, D.A., 2003. Computational modeling of the EGF-receptor system: a paradigm for systems biology. Trends Cell Biol. 13, 43–50.

Wang, Z., Moran, M.F., 1996. Requirement for the adapter protein GRB2 in EGF receptor endocytosis. Science 272, 1935–1939.

Whitmarsh, A.J., Davis, R.J., 1998. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem. Sci. 23, 481–486.

Wagner, B.J., Hayes, T.E., Hoban, C.J., Cochran, B.H., Doolittle, R.F., 1990. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J. 9, 4477–4484.

Wang, D., DuBois, R.N., 2006. Prostaglandins and cancer. Gut 55, 115–122.

Yarden, Y., Schlessinger, J., 1987. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry 26, 1443–1451.

Yip, C.C., Ottensmeyer, P., 2003. Three-dimensional structural interactions of insulin and its receptor. J. Biol. Chem. 278, 27232–27329.

Yamanaka, M., Ishitani, R., Nureki, O., et al., 2002. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell 100, 775–787.

Yun, C.-H., Boggon, T.J., Li, Y., Woo, M.S., Greulich, H., Meyerson, M., Eck, M.J., 2007. Structure of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell 11, 217–227.

Yao, Y., Li, W., Wu, J., Germann, U.A., Su, M.S., Kuida, K., Boucher, D.M., 2003. Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. PNAS U.S.A. 100, 12759–12764.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W., 1999. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature 401, 173–177.

Yang, Q., Liao, L., Deng, X., Chen, R., Gray, N.S., Yates, J.R., Lee, J.D., 2013. BMK1 is involved in the regulation of p53 through disrupting the PML-MDM2 interaction. Oncogene 32, 3156–3164.

Zarogoulidis, K., Zarogoulidis, P., Darwiche, K., Boutsikou, E., Machairiotis, N., Tsakiridis, K., Katsikogiannis, N., Kougioumtz, I., Karapantzos, L., Huang, H., Spyratos, D., 2013. Treatment of non-small cell lung cancer (NSCLC). J. Thorac. Dis. 5, S389–S396.

Zhong, Z., Wen, Z., Darnell, J.E.J., 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 264, 95–98.

Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K., Ullrich, A., 1997. Critical role of calcium- dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. J. Biol. Chem. 272, 24767–24770.

CHAPTER

11

Signal Transduction to and from Adhesion Molecules

In this chapter, we consider the adherence of cells to surfaces or to other cells and ask how this affects their responses to soluble agonists such as growth factors. Also, how soluble agonists affect cellular adherence, itself a signaling event important in the maintenance of stem cell compartments and the epithelial mesenchymal transition (discussed in Chapter 12, "Wnt Signaling and the Regulation of Cell Adhesion and Differentiation"). The molecules that effect adhesion, serve both as targets for signals that are generated within cells (inside-out signaling) and as receptors for extracellular signals (outside-in signaling). These two aspects are well exemplified in the regulation of survival, proliferation, differentiation, and in leukocyte trafficking (discussed in the Chapter 14, "Chemokines and Traffic of White Blood Cells"). This list is far from complete. Adhesion molecules are of prime importance in the functioning of synapses, nerve cell and keratinocyte differentiation, gene expression in epithelial cells, thymic selection, and activation of T lymphocytes.

ADHESION MOLECULES

Initially, adhesion molecules were described innocently as a sort of glue, sticky stuff having no other functional implications. We now recognize that they also act as signaling molecules and are properly described as receptors (Figure 11-1). However, the ligands that interact with adhesion molecules are generally insoluble, frequently adhesion molecules themselves, presented by adjacent cells or components of the extracellular matrix (juxtacrine communication mode).

Adhesion molecules first came to light as a result of investigations of brain development around 1970. It was realized that the very precise organization of neural cells in the central nervous system must require a dynamic process of cell guidance and cell adhesion. This would drive the

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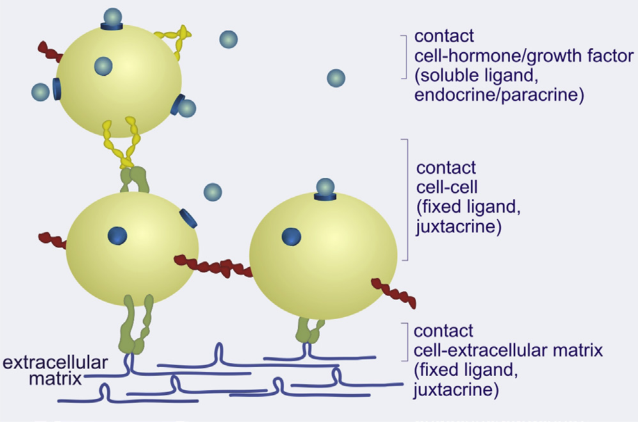


FIGURE 11-1 Three levels of communication. Cells are in contact with soluble ligands (hormones and cytokines, endocrine or paracrine communication), with each other and with the extracellular matrix (juxtacrine communication). The latter contacts are mediated by adhesion molecules, which in many instances, should be regarded as receptors that convey signals into the cells.

direction-seeking processes of neurite outgrowth and synapse formation. Two main ideas were considered (Edelman, 1983). The first suggested that during development, in order to establish precise cell–cell contacts, the interacting cells must each present unique adhesion molecules that fit each other as a lock and key (chemoaffinity hypothesis). The second idea was that the set of adhesion molecules is limited, but their binding capacity could be modulated over time. For instance, developing neuronal cells would all offer the same molecule and during outgrowth, this would be in a low-affinity state. The cell might then convert the adhesion molecule into its high-affinity state, to promote binding to its counterpart on a nearby cell.

It now appears that there is truth in both these propositions. The number of adhesion molecules is certainly limited and their capacity to interact with counter receptors is regulated by their levels of expression (presence or absence) and also by their binding state (affinity). In the realm of immunology, the set of adhesion molecules expressed on a cell surface and their state of activation has been called the “area code” (Springer, 1994). The area code defines where blood-borne cells leave the circulation and enter the tissue.

Clear evidence for specific adhesion interactions came from studies of the reaggregation of disaggregated tissues. Tissue cells dispersed by treatment with trypsin, which strips proteins from the surface, only recover their adherent properties after a period in culture. The need for a recovery period suggested that new adhesion molecules must be

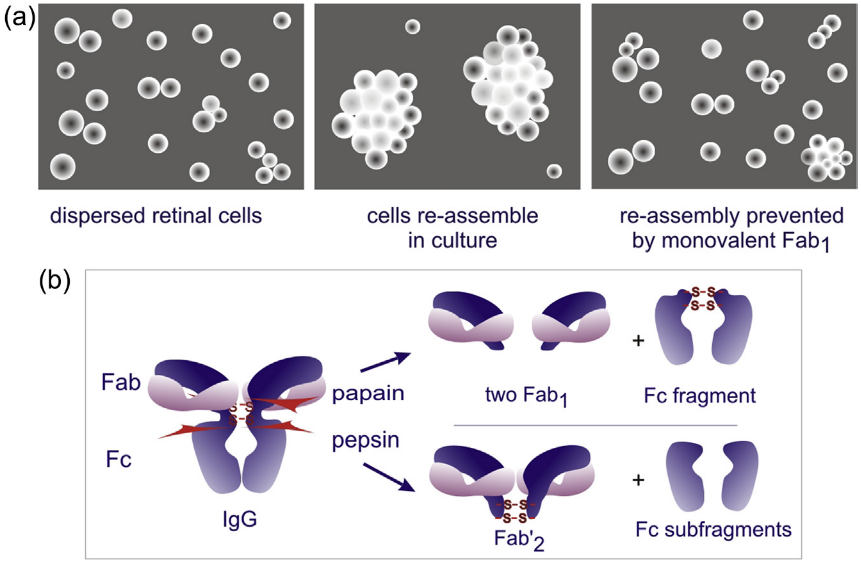


FIGURE 11-2 Reassembly of retinal-dispersed cells: discovery of N-CAM. (a) The reassembly of dispersed cells is prevented by antibodies that recognize cell-membrane proteins. By this approach, the role of the adhesion molecule NCAM in the maintenance of tissue integrity was discovered. Since intact bivalent antibodies would have forced the cells to aggregate it was essential to use monovalent Fab fragments that are unable to form cross-links. *(Image adapted from Brackenbury et al. (1977).)* (b) Generation of the fragments Fc, Fab_1 (monovalent) and Fab_2 (divalent) by enzyme digestion of immunoglobulin G.

expressed on the cell surface. The reaggregation can be prevented by monovalent Fab_1 fragments prepared from antibodies raised against the cell membranes, which block cell-surface epitopes (Figure 11-2). Because such binding sites could never be saturated by a few micrograms of "blocking" antibodies the possibility of weak nonspecific interactions was precluded and it followed that there is a limited number of specialized molecules that determine cell–cell interactions. These are the adhesion molecules and the first to be discovered was NCAM (Neuronal Cell Adhesion Molecule) (Brackenbury et al., 1977).

Divalent antiserum (intact immunoglobulins with two antigen-binding sites) would have the effect of aggregating the cells. The use of monovalent Fab fragments also ensures that no signals ensue from the crosslinking of surface molecules that might follow the use of intact divalent antiserum.

Complement is the term originally used to refer to the heat labile factor in serum that causes immune cytolysis, the lysis of antibody-coated cells and now referring to the entire functionally related system comprising at least 20 distinct serum proteins that is the effector not only of immune cytolysis but also of other functions.

Early attempts to identify individual adhesion molecules were hampered by the lack of specific antibodies. The key was provided by the advent of monoclonal antibodies and their application to questions regarding cell adhesion. This led to the identification of Mac-1, expressed on the surface of macrophages and that which plays a pivotal role in the binding of leukocytes to the vascular endothelium (Springer et al., 1979). It also determines the binding of the serum complement factor C3bi, which, together with the Fc receptor, mediates activation of the respiratory burst. Later, a monoclonal antibody that recognizes LFA-1 (Lymphocyte Function-associated Antigen-1) was used to show that the binding of cytotoxic T-lymphocytes to their target cells is also mediated by specific adhesion molecules (Davignon et al., 1981).

Adhesion molecules not only link cells to surfaces but they also make the connection between the extracellular matrix and the cytoskeleton. Because it was perceived that they integrate intracellular and extracellular events they were called integrins (Tamkun et al., 1986). These proteins can bind to a wide range of extracellular matrix molecules, including fibronectin, fibrinogen, laminin, osteopontin, thrombospondin, vitronectin, and von Willebrand factor. A number of integrins bind these proteins through the recognition of short amino-acid motifs such as -RGD- or -EILDV- (Ruoslahti and Pierschbacher, 1987) Mac-1 and LFA-1; share substantial sequence homology with the integrins; and it was confirmed that they too are members of this family of adhesion molecules.

NAMING NAMES

Adhesion molecules have been given names that express function (intercellular adhesion molecule-1, ICAM-1), location and function (endothelium leukocyte adhesion molecule-1, ELAM-1), the need of Ca^{2+} for their binding (Ca^{2+}-dependent adherence, cadherins), the time of induction of expression during T-cell activation (very late antigen-4, VLA-4), or in recognition of their integration of the extracellular matrix with the intracellular cytoskeleton (integrins). Of course, there are other names, often bestowed after cloning (αM integrin) or through recognition by specific monoclonal antibodies (CD11b, cluster of differentiation 11b). If this is not all perfectly clear, then one should appreciate that CD18/CD11b is synonymous with Mac-1, which is synonymous with integrin αMβ2 (gene names ITGAM and ITGB2) and that CD62E is synonymous with ELAM-1, which is synonymous with E-selectin (gene name SELE). αLβ2 is synonymous with LFA-1 (gene names ITGAL and ITGB2). Such are the problems of nomenclature when molecular cloning rubs shoulders with immunology, pharmacology, and all the rest. Throughout the chapter we mention the approved gene names (HUGO Gene Nomenclature Committee).

IMMUNOGLOBULIN SUPERFAMILY

NCAM is a member of a very large family of cell surface proteins that express repeated immunoglobulin or immunoglobulin-like domains at their extracellular N-termini. Proteins belonging to the immunoglobulin superfamily and that play a role in adhesion are called Ig-cellular adhesion molecules. The immunoglobulin structures, classified as antiparallel, Greek key, β-sandwiches, are stabilized by sulfydryl bridges and are resistant to proteases (Figure 11-3, left panel). These immunoglobulin domains are then subclassified as C1-set, C2-set, V-set, I-set, or Ig-like, based on their similarity to the constant, variable, or intermediate sequence patterns in immunoglobulins (“antibodies”) (Smith and Xue, 1997). Ig-cellular

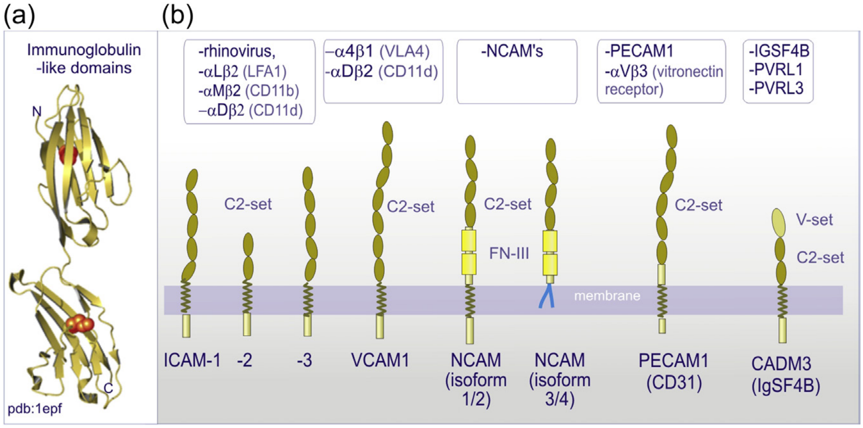


FIGURE 11-3 Adhesion molecules of the immunoglobulin superfamily. (a) Ig family adhesion molecules are characterized by repeated domain structures that are homologous to those present in immunoglobulins (Ig domains). The disulphide bonds are presented as red spheres (left panel). (b) There are several members of this family, all having a single membrane-spanning domain. They interact with different ligands (or counter receptors). NCAM isoforms three and four are attached to the membrane by a glycosyl–phosphatidyl inositol anchor.

adhesion molecules can be arranged in subfamilies based on the type of immunoglobulin domain they contain (C1-type, C2-type, etc.). However, since many carry multiple different domains it proved to be impossible to classify them systematically and we will only elaborate on a few examples because the list is otherwise too vast to be meaningful.

For all adhesion members of the immunoglobulin superfamily (IGSF) we suggest to explore the HUGO gene nomenclature committee Web site. URL: www.genenames.org/genefamilies/IGSF.

VCAM1 (vascular cell-adhesion molecule-1, existing in two splice variants with five or seven Ig domains) (Osborn et al., 1989), NCAM (four splice variants), PECAM1 (platelet endothelial cell-adhesion molecule, CD31), and CADM3 (cell-adhesion molecules also known as immunoglobulin superfamily member 4B precursor, IgSF4B), present in brain synapses, contain two C2- and one V-set Ig domain (Figure 11-3). VCAM-1 is highly expressed on endothelial cells and certain fibroblasts when present in inflamed tissues. VCAM-1 binds integrins α4β1 (VLA-4) and αDβ2.

Intracellular adhesion molecule

ICAMs form a small family of their own, ICAM 1-5 (Dustin et al., 1986), all containing varying numbers of Ig-set C2-type domains. They bind the integrin αLβ2 (LFA-1) and then a diverse range of other ligands/counter receptors. ICAM-1 and -2 are important for the recruitment of leukocytes into the tissues. Upon binding of LFA-1, they signal into the cell through members of the Rho family of GTPases. This mediates subsequent transendothelial migration through the loosening of the VE-cadherin cell–cell contact sites and by rearrangement of the actin cytoskeleton (Wojciak-Stothard and Ridley, 2003; Barreiro et al., 2002). The interaction with the actin cytoskeleton is mediated through members of the ERM family (ezrin/moesin/radixin). All this will be further elaborated in Chapter 14, "Chemokines ad Traffic of White Blood Cells."

ICAM-2 on vascular endothelial cells also supports homophilic interactions that may be involved in vascular tube formation during the process of angiogenesis (formation of vasculature from a preexisting vascular bed (Huang et al., 2005)). ICAM-3 is restricted to leukocytes (de Fougerolles and Springer, 1992) and ICAM-4 to red blood cells, constituting the Landsteiner–Wiener blood group (Bailly et al., 1994). ICAM-5 is expressed in neurons (Kilgannon et al., 1998).

Sialic acid-binding Ig-like lectin protein

The SIGLECs (sialic acid-binding Ig-like lectin proteins) constitute a subfamily within the superfamily of immunoglobulins. The majority is ranked among the V-set domain-containing members of the immunoglobulin super family (IGSF) (Table 11-1). Members of the SIGLEC subgroup include SIGLEC1 (sialoadhesin), CD22 (Siglec-2), CD33 (Siglec-3), MAG (Siglec-4), and SIGLEC5–11, 14, 15 (13 does not exist in humans and 12 and 16 qualify as pseudogenes). They are characterized by the presence of a single N-terminal Ig V-set domain, which has the characteristics of an "I-type lectin" that binds sialic acid (see also below "selectins") (Powell and Varki, 1995). Anything between 1 and 16 Ig-like C2-set domains project this sialic acid-binding site away from the plasma membrane. Each SIGLEC has a preference for a specific type of sialic acid and for a specific type of linkage to the subterminal sugar. The cytosolic tails vary in sequence and length but most have conserved tyrosine residues within immunoreceptor tyrosine-based inhibition motifs (ITIMs). These motifs play a role in signaling. For CD22 it was shown to bind the SH2 domain-containing tyrosine phosphatase SHP1 (gene name PTPN6). SIGLEC 14 and 15 form an exception as they lack the ITIM motif and instead carry a lysine or an arginine that binds an ITAM adaptor (DAP10 or DAP12).

TABLE 11-1 Human SIGLEC adhesion molecules

Human siglec	Ig-domains	Expression	IGSF subfamily
SIGLEC1	16C2, 1 V	Macrophage	V-set
CD22 (Siglec-2)	6C2, 1 V	B-cell	C2-set
CD33 (Siglec-3)	1C2, 1 V	Monocyte, myeloid progenitor	Ig-like
MAG (Siglec-4)	4C2, 1 V	Oligodendrocyte, Schwann cell	C2-set
SIGLEC5	2C2, 1 V	Monocyte, neutrophil, B-cell	V-set
SIGLEC6	2C2, 1 V	B-cell, placental trophoblast	V-set
SIGLEC7	2C2, 1 V	Monocyte, NK cell	V-set
SIGLEC8	2C2, 1 V	Eosinophil, basophil, mast cell	V-set
SIGLEC9	2C2, 1 V	Monocyte, neutrophil, NK cell	V-set
SIGLEC10	3C2, 1 V	Monocyte, NK cell	V-set
SIGLEC11	3C2, 1 V	Macrophage	V-set
SIGLEC14	2C2, 1 V	Unknown	V-set
SIGLEC15	2C2, 1 V	Unknown	V-set

Instead of a phosphatase, they recruit the regulatory subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3R1) and the adaptor protein GRB2 (Crocker et al., 2007; Angata et al., 2007). SIGLEC1 and MAG lack a specific motif.

SIGLEC adhesion molecules are present in myeloid cells (granulocytes, lymphocytes, and monocytes), Schwann cells, and placental trophoblasts. They are not expressed in *Drosophila* or *Caenorhabditis elegans* probably representing a later adaptation of an ancient protein-binding Ig domain (see Figure 11-4).

The term "sialic acid" is derived from the Greek *sialos*, meaning "saliva" where it was first identified. They are sugars having nine carbon atoms.

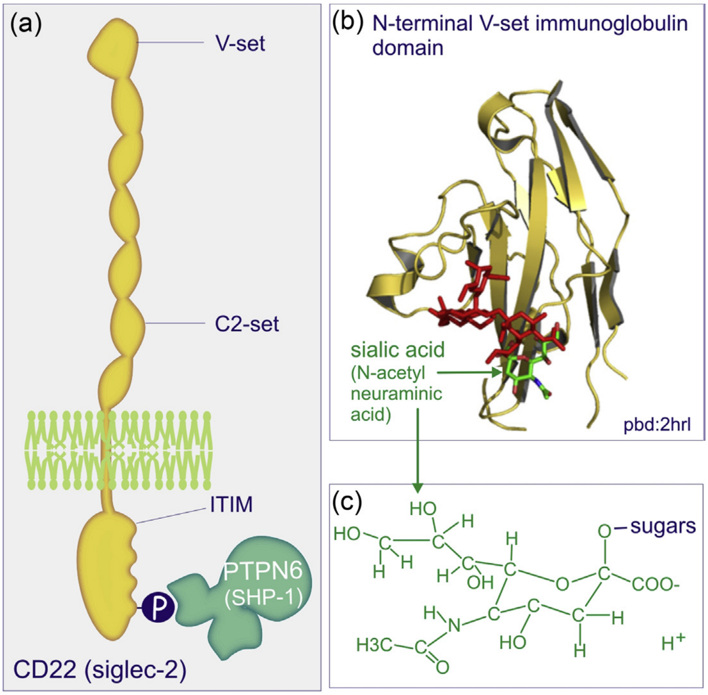


FIGURE 11-4 Structure of CD22/SIGLEC-2. (a) The extracellular domain of CD22 is composed of six Ig C2-set domains with an N-terminal V-set domain that binds sialic acid. The cytoplasmic segment contains four ITIM motifs with tyrosine phosphorylation sites. One of these is recognized by one of the two SH2 domains of the phosphatase PTPN6. (b) Details of the binding of *N*-acetylneuraminic (a sialic acid, green sticks) to the immunoglobulin V-set domain of the mouse SIGLEC7. (c) Structure of sialic acid (*N*-acetyl neuraminic acid).

Of the many SIGLECs, CD22, a single-span membrane protein expressed on cells of B-cell lineage and which recognizes sialic acid linked to galactose, has attracted much attention. This is because it is an inhibitory co-receptor that down-modulates signaling through the B-cell receptor (BCR). It does this by setting a threshold that prevents overstimulation, important in the maintenance of tolerance to some antigens. In mice, disruption of the CD22 gene gives rise to a hyperresponsive BCR and the animals exhibit an augmented immune response (O'Keefe et al., 1996).

CD22 binds to sialic acid residues that are attached to the B cell itself (*in cis*) as well as those that are attached to other cells that present antigens *(in trans)*. Binding *in cis* keeps CD22 away from the BCR and enhances signaling (Collins et al., 2006). When this is prevented, for instance through binding to sialic acid residues of an adjacent antigen-presenting cell or by binding directly to the "engaged antigen" receptor, it inhibits BCR signaling. The reason for this is that the activated BCR recruits the LYN kinase that phosphorylates the adjacent tyrosine residues in the three ITIM motifs of CD22 (Nishizumi et al., 1998). This in turn engages the tyrosine phosphatase PTPN6 that prevents BCR signaling by dephosphorylation of its own phosphotyrosyl residues, present in the ITAM motifs (see Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases") (Nitschke, 2005).

We note that SIGLEC1 and -5 bind to sialylated lipopolysaccharides expressed by *Neisseria meningitidis* (a bacteria that causes meningitis) and that this enhances phagocytosis (uptake) by macrophages (Jones et al., 2003). Likewise, glycoprotein-B expressed on the varicella-zoster virus (causing initially chickenpox in youth and shingles at later stages) bind to MAG (Siglec-4) and this promotes membrane fusion, a process that allows the viruses to enter the cell (infection) (Suenaga et al., 2010).

Junctional adhesion molecule (JAM)

The tight junction, originally identified in the early 1960s by Farquhar and Palade, (Farquhar and Palade, 1963) is an extensive intramembrane multiprotein complex that provides apical intercelluar connections between adjacent cells in both epithelial and endothelial monolayers. The junctional adhesion molecule (JAM) constitutes a further subgroup of the immunoglobulin superfamily. Four members are expressed in humans, F11R (JAM-A), JAM2, JAM3, and IGSF5 (JAM4) (the first three are arranged among the V-set domain-containing IGSF members, the last one ranks among the Ig-like subset). They are single-span membrane proteins having two extracellular immunoglobulin domains of V-, C2-, or I-set. They possess C-terminal motifs that interact with tight junction proteins of the tight junction protein, TJP, family (TJP1–3, also known as zonula occludens ZO1–3) (Figure 11-5). They contribute to the architecture of

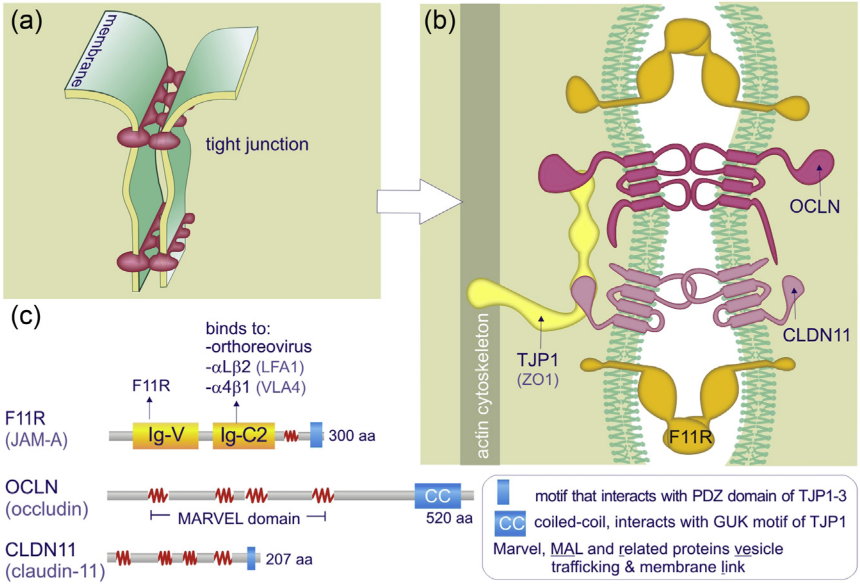


FIGURE 11-5 Tight junction proteins, JAM, occluding, and claudin. (a) Diagram illustrating a tight junction as present in endothelial and epithelial tissues. (b) The junctions are composed of three types of proteins. F11R (JAM-A) is a member of the immunoglobulin superfamily of adhesion molecules. CLDN11 (claudin) and OCLN (occludin) form separate groups, both spanning the membrane four times and having very short extracellular domains. Their homophilic interaction ensures close apposition of the two membranes. There are many variants of the CLDN (24 genes). The combination expressed in tight junctions determines the accessibility of the paracellular space. They all bind to TJP1 (or −2 or −3) which connects the junctional proteins to the actin cytoskeleton. (c) Domain architecture of proteins of the tight junctional proteins.

tight junctions in epithelial and endothelial cells and are also expressed on lymphocytes, megakaryocytes, platelets, and red blood cells. Both homo- and heterophilic interactions have been reported. Importantly, they play a role in viral infection, serving as a receptor for orthoreoviruses, and in leukocyte transendothelial migration by binding to αLβ2 or α4β1 integrins (see Chapter 14, "Chemokines and Traffic of White Blood Cells," section "Migration within the tissues").

CLAUDINS

The claudins form a large group of adhesion molecules (23 members in human (designated CLDN1 to −23)) of 23 kDa, with four-span topology having short cytosolic N- and C-terminal domains (Figure 11-5). They are not members of the immunoglobulin superfamily but they belong to a family of PMP22/EMP/MP20/claudin proteins, all with the same membrane topology and a characteristic signature motif in the first extracellular loop (-(L/V)WxxC-). The claudins contribute to the selectivity of paracellular transport (meaning transport between, not through, cells). Thus, they are the principal barrier-forming proteins of the tight junction and their expression pattern determines the barrier qualities of epithelial sheets. Their expression patterns make the distinction between those epithelia having high resistance and which maintain strong ionic gradients (such as the distal nephron and urinary bladder), and leaky epithelia having low resistance, and which allow the movement of large volumes of iso-osmotic fluid (as occurs in much of the gastrointestinal tract Turksen and Troy, 2004; Van Itallie and Anderson, 2006). The C-terminus contains a short sequence motif that binds the PDZ domain in adaptor proteins such as TJP1–3 (Itoh et al., 1999).

Tight junctions are also referred to as zonula occludens. The word occludens comes from Latin “occludere,” meaning “locked up.” Claudin is derived from the latin “claudere,” meaning “close.”

CCLDN3 and -4 act as receptors for the bacterial endotoxin CPE produced by *Clostridium perfringens*. This causes a profound change in the structure of the tight junctions of the enterocytes, followed by an increased paracellular leakage of water. The failure to reabsorb water in the colon results in diarrhea.

OCCLUDINS

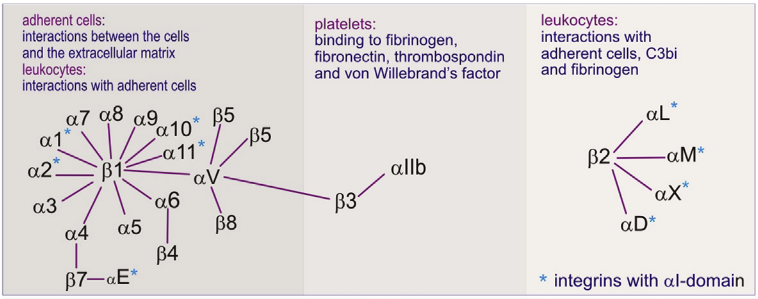
Occludin (OCLN) is a four transmembrane-spanning protein of 60 kDa having a short N-terminal and a much longer C-terminal region both exposed in the cytosol. Only a single gene exists. It belongs to the group of tight junction-associated MARVEL domain-containing proteins characterized by four transmembrane-spanning segments that share homology with the MAL protein (Sanchez-Pulido et al., 2002). Other group members that occur in tight junctions are tricellulin and MarvelD3 (van Itallie and Anderson, 2014). It is a component of tight junctions in endothelial and epithelial cells (Figure 11-5). Its typical apical localization occurs through interaction between its coiled-coil domain (C-terminal segment) and TJP1, which are linked to the actin cytoskeleton. Although only one gene exists, the protein nevertheless occurs in many forms because of alternative splicing and numerous posttranslational modifications. These range from proteolysis, phosphorylation, dimerization, to ubiquitylation. A small fraction of occludin is detected on the lateral membrane (van Itallie and Anderson, 2014; Cummins, 2012).

Web resource

Kyoto encyclopedia of genes and genomes, pathway map 04530 "tight junction," URL: www.genome.jp/dbget-bin/www_bget?map04530.

INTEGRINS

The integrins are the most dynamic and versatile of the adhesion molecules. They are composed of two subunits (α and β), linked non-covalently. There exist 18 α- and 8 β-subunits and these can form at least 23 different integrin heterodimers (Figure 11-6). Depending on the particular αβ combination, they may bind to ICAMs, VCAM-1, or MadCAM



examples:
cell-to-extracellular matrix
α7b1 -- laminin
αVβ3 -- vitronectin/fibronectin/osteopontin
α5β1 -- fibronectin
α6β4 -- laminin
α1β1 -- collagen, laminin

leukocytes-to-adherent cells
α4β1 (VLA4) -- VCAM-1, F11R (tight junction)
α4β7 -- MadCAM

cell-to-cell
αLβ2 (LFA1) -- ICAM1, ICAM2, ICAM3, F11R
αMβ2 (CD11b) -- ICAM1
αDβ2 (CD11d) -- ICAM-3, VCAM-1,

cell-to-extracellular matrix
αMβ2 -- C3bi , fibrinogen
αXβ2 -- fibrinogen

gene names
α1 - ITGA1, αV - ITGAV, αX - ITGAX, etc
β1 - ITGB1, β2 - ITGB2, etc

FIGURE 11-6 Mixing and matching integrin subunits. Various combinations of α- and β-subunits create integrins having specific ligand-binding characteristics. The β2-integrins are restricted to blood cells, β1 are present on all cells. Platelets have uniquely the combination of all β3, vital in blood clotting. Integrins bind to specific sequences in proteins of the extracellular matrix such as Arg-Lys-Asp (-RGD-) or Glu-Ile-Leu-Asp-Val (-EILDV-).

(on mucosal cells). They may also bind to components of the extracellular matrix such as collagen, fibronectin, laminin, or vitronectin and to the blood proteins, fibrinogen or von Willebrand factor.

The subunits of the integrins (α and β) are transmembrane glycoproteins having a head, stalk, and a short (40–60 amino acids) cytoplasmic region. Integrin β4, which is specialized to connect to the keratin cytoskeleton in junctional complexes with the basement membrane (hemidesmosomes), has a more extended cytoplasmic region. The stalk is composed of a number of highly conserved domains as depicted in Figure 11-7. As will become evident, the positioning of the "hybrid domain" within the stalk region of the β-integrin subunit has an

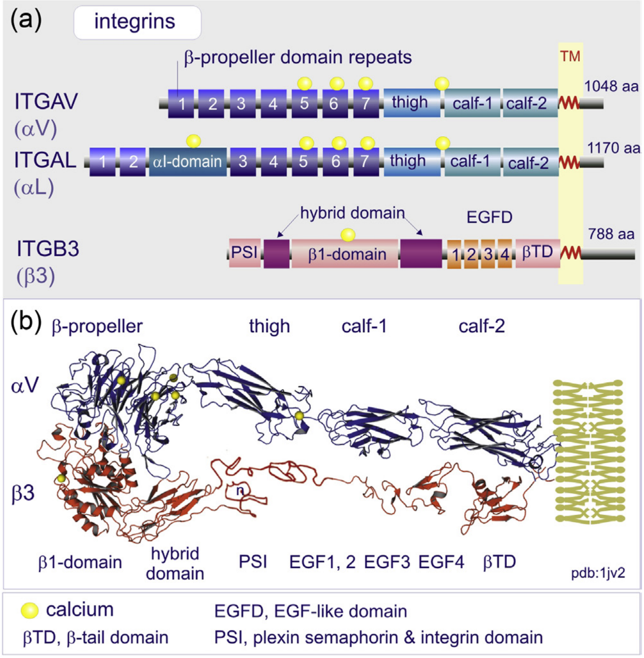


FIGURE 11-7 Integrins. (a) Domain architecture of integrins. (b) Integrins are composed of two non-covalently bound subunits—α and β. They are involved in cell–cell and cell–extracellular matrix contact and are linked to the actin cytoskeleton. They are transmembrane proteins composed of head, stalk, and cytoplasmic segments. The stalk segment of the α-subunit comprises two calf domains and a thigh domain. The protein articulates at the border of the calf-1 and thigh domain. The PSI and hybrid domain of the β-subunit are pushed outward in the switch blade movement and this plays an important role in activation process. The heads contain various subdomains of which the β-propeller (on the α-subunit) and β1-domain (on the β-subunit) assure the association of the two subunits. Depending on the type of integrin, ligand recognition either occurs through the β1-domain or through the αI-domain (compare domain architecture of αV with αL).

essential role in regulating the affinity for ligand. The head regions of β-integrin subunits all have a β1-domain (also referred to as the I-like domain) while the head regions of α-integrin subunits vary according to the type of integrin. They all contain a β-propeller structure but some have an αI-domain insert (these integrins are indicated with an asterisk in Figure 11-6). Two examples of the different α-subunits are presented in the top panel of Figure 11-7 (compare αV with αL). Importantly, the αI- and the β1-domains have similar folds and can both interact with ligand.

The β-propeller structure and the β1-domain are responsible for linking the α- and β-subunits (Shimaoka et al., 2002). This interaction resembles the linking of α- and β-subunits of heterotrimeric GTP-binding proteins (return to Chapter 2, "An Introduction to Signal Transduction," Figure 2-30). The β1-domain is a mutational hotspot. This can result in a failure of association and can give rise to leukocyte adhesion deficiency, characterized by frequent unresolved infections (see Chapter 14, "Chemokines and Traffic of White Blood Cells").

The FERM domain (present in erythrocyte band 4.1, ezrin, radixin, and moesin) is widespread, composed of ~300 amino acids present in a number of proteins that operate at the interface between membranes and the actin cytoskeleton. Apart from the proteins mentioned in this section, the FERM domain is found in focal adhesion kinase (FAK), janus tyrosine kinase (JAK), non-receptor tyrosine kinase (TYK), and some protein tyrosine phosphatases.

Inactive to primed integrins

Integrins can exist in low-affinity (inactive), high-affinity (primed), or ligand-bound (activated) states, determined by conformational changes in the head region.

The shift from low to high affinity is directed from inside the cell by association of proteins with the cytosolic region of the α- and β-subunits (inside-out signaling). Essential in the process is the disruption of a salt bridge between transmembrane helices of the (inactive) α- and β-integrin subunits, which leads to an altered orientation of the transmembrane region of the β-subunit. This then alters the stalk region, which changes from a bent to an extended conformation, referred to as the "switch-blade upward movement" (panel (a), Figure 11-8). Talin (TLN1), a large cytoplasmic protein comprising a head and a rod segment, plays an essential role in all this. Its head contains an atypical FERM domain comprising F0 to F3. Of these F3 binds the cytoplasmic tail of

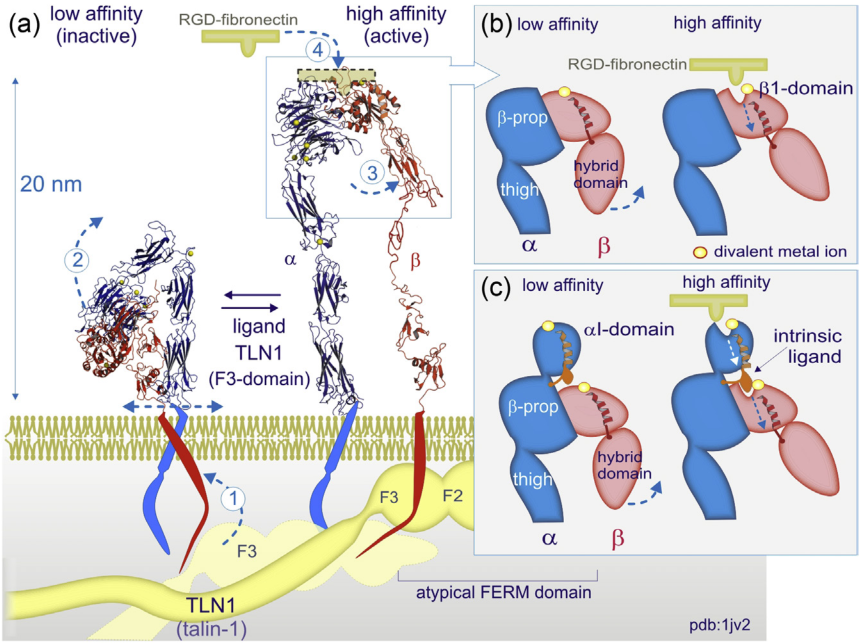


FIGURE 11-8 Mechanisms of integrin activation. (a) The inactive integrin is in a folded state, head down. (1) Binding of talin-1, through the F3 region in the FERM domain disrupts a salt bridge between the transmembrane helices of the (inactive) α- and β-integrin subunits. This leads to an altered orientation of the transmembrane region of the β-subunit which effectively sets the transmembrane domains apart. (2) The separation causes a switch-blade upward movement which is followed (3) by a change in the positioning of the hybrid domain of the β-subunit, which moves to the outside position. (4) The integrin is now ready to bind ligand (primed state). (b) The outward movement of the hybrid domain changes the conformation of the β1-domain, creating a ligand-binding pocket. (c) For those integrins that carry an αI-insert, the mechanism of activation is identical but the change in conformation is indirect. The movement of the hybrid domain creates an "intrinsic ligand-"binding site which, when occupied, causes a conformational change in the αI-domain, creating a second ligand-binding pocket, this time for the extracellular matrix (fibronectin in the example). *Source: Composition based on information in Shimaoka et al. (2002), Xiong et al. (2001), Xiao et al. (2004), and Carman and Springer (2003).*

β-integrins (involving among other amino acids a characteristic -NPxY- motif) and this brings about the change in position of the transmembrane domain. Indeed, addition of purified FERM domains suffices to cause the switch-blade movement of integrins when embedded in membrane discs (Tanentzapf and Brown, 2006; Ye et al., 2010. We return in more detail to the intracellular events in the section "integrins, cell survival, and cell proliferation," Figure 11-14).

Talin was first identified in membrane ruffles and cell–extracellular matrix junctions (focal adhesions) in 1983. The discovery that it bound two other focal adhesion proteins, namely vinculin and integrins, attracted much interest (Burridge and Connell, 1983). Initial evidence that talin is important for focal adhesion assembly came from antibody microinjection, gene downregulation, and gene deletion experiments (reviewed in Critchley, 2009). The first mechanistic insights into talin function came in around 2000 from the landmark discoveries that the talin head binds directly to the cytoplasmic tails of β-integrin subunits and performs the key final step in integrin activation (Calderwood et al., 1999; Tadokoro et al., 2003).

The movement of the stalk region and its effect on the head structure has been studied in great detail for integrin αIIbβ3 (also known as glycoprotein IIb/IIIa), expressed on platelets and involved in the clotting process through binding to fibrinogen (Xiao et al., 2004). As the stalk region straightens, there is a movement of the "hybrid domain" of the β-subunit, which causes a shape change of the neighboring β1-domain (panel (b), Figure 11-8). This exposes the ligand-binding site and converts the integrin into a primed, high-affinity, adhesion molecule. Similar changes occur on those integrins that carry an αI-insert (αLβ2), but here the change has to be propagated by means of an "intrinsic ligand" (panel (c), Figure 11-8) (Law et al., 2004; Carman and Springer, 2003). The integrins of circulating cells such as leukocytes are maintained in an inactive state and are stimulated by chemokines acting through G-protein-coupled 7TM receptors. In contrast, for tissue cells the integrins appear to be constitutively activated, though local inactivation can occur, as must be the case in migrating cells.

Primed to active

Ligand binding causes further alterations in the relative positioning of the stalk regions (Mould and Humphries, 2004) so that they recruit proteins that constitute an intracellular signaling complex. Some 50 proteins have been implicated in the localization (transient or stable) of these focal adhesion complexes (Miranti and Brugge, 2002; Miyamoto et al., 1995). It is not yet clear which proteins actually sense the ligand-bound conformational change and pass the message on into the cell. The clustering of integrins that follows ligand binding (formation of focal adhesion contacts) probably plays an important part in the formation of intracellular signaling complexes, though clustering alone fails to reproduce full outside-in signaling events (Hato et al., 1998). Finally, among the cytosolic proteins that localize in the focal-adhesion complex, are ILK (integrin-linked kinase), focal-adhesion kinase (PTK2), BCAR1, α-actinin (ACTN1), talin (TLN1), paxillin (PXN), and vinculin (VCL). These are all multi domain proteins that link integrins with components of the actin cytoskeleton (Brakebusch and Fassler, 2003) and with signaling proteins such as protein kinases and GTPases. Once again, integrin β4 present in hemidesmosomes is an exception (see above), being coupled to intermediate filaments through plectin (PLEC).

CADHERINS

In the formation of the early embryo, the compaction of the morula is mediated through Ca^{2+}-dependent adhesion molecules (Ducibella and Anderson, 1975) (Figure 11-9). Uvomorulin, one of the first to be identified, is instrumental in the transition from a grape-like to a mulberry-like object. The cellular junctions thus formed are linked by a contractile network of actin filaments that by shortening, pulls the embryonic cells together in a manner similar to a purse-string. Compaction not only tightens the bonds between the cells, it also introduces, for the first time, morphological polarity. From hereon, embryonic cells have a smooth basal surface on one aspect and an apical surface dotted with microvilli on the other.

A morula (from Latin "morus" meaning mulberry) is an embryo at an early stage of embryonic development, consisting of approximately 8–32 cells (called blastomeres). During compaction, the blastomeres change their shape and tightly align with each other to form a compact ball of cells. This process is mediated by adhesion molecules, cadherin being one of the most important.

Investigations using teratocarcinoma F9 embryonal carcinoma cells have revealed a whole range of Ca^{2+}-dependent adhesion molecules, collectively the cadherins (Yoshida and Takeichi, 1982). They comprise a large family (at least 36 members in humans) mediating homotypic cell–cell adhesion, acting as both receptor and ligand. The first to be discovered were E-cadherin (also named L-CAM, for liver cellular-adhesion molecule) (Gallin et al., 1983) and uvomorulin (Boller et al., 1985). They proved to be one and the same protein, cadherin-1 (gene name CDH1). Many cadherins were initially named after the tissue in which they were discovered (epithelial, placental, neuronal, etc.), but these labels have little

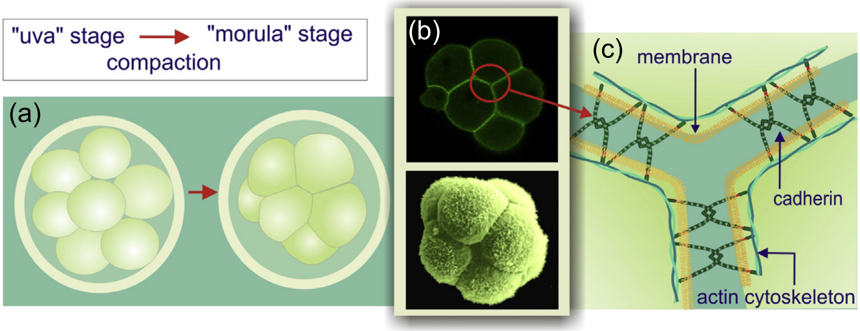


FIGURE 11-9 Role of cadherin in the compaction of the eight cell-stage mouse embryo. Scanning electron microscopy reveals that after three cell divisions (eight cell-stage) mouse embryos change from a uva (grape)-like to a morula (mulberry)-like aggregate. This process, that is called compaction, enables the cells to attach firmly to each other, manifesting first signs of polarization. Their morphology now presents distinct basal (contact) and apical (peripheral) membrane surfaces. Cadherins play an important role in the compaction process, they are localized at cell–cell boundaries and their link to the actin cytoskeleton allows both the compaction of the embryo and the profound shape change of the cells. *Images in center panel are the courtesy of Henri Alexandre.*

meaning since most of them are more widely expressed. The individual cadherins do, however, show restricted and distinct expression patterns.

Cadherins are calcium-dependent transmembrane proteins that generally mediate cell–cell adhesion or cell–cell recognition and are characterized by the presence of at least two consecutive extracellular cadherin-specific domains, called cadherin repeats (EC), comprising some 110 amino acids with conserved calcium-binding sites (panel (b), Figure 11-10). These modules are numbered, starting with EC1 at the N-terminus. Some cadherins have as many as 34 of these repeats, though most have only four or five. The cadherin superfamily can be phylogenetically divided into three major families: the major cadherin family, the protocadherin family, and the cadherin-related family.

All members of the major cadherin (CDH) (http://www.genenames.org/cgi-bin/genefamilies/set/18) gene family encode proteins with an extracellular segment comprising at least five consecutive cadherin repeats showing high sequence conservation within the family. The family includes the "classical" type I and type II cadherin genes, encoding proteins with a single transmembrane domain and a cytoplasmic domain containing two conserved motifs for binding to members of the armadillo (β- and δ-catenin) protein family. Further, the family includes type-III, type-IV, and desmosomal cadherins, often encoding longer extracellular segments with additional motifs, a single transmembrane domain, and a cytoplasmic domain containing at least one conserved motif for armadillo protein

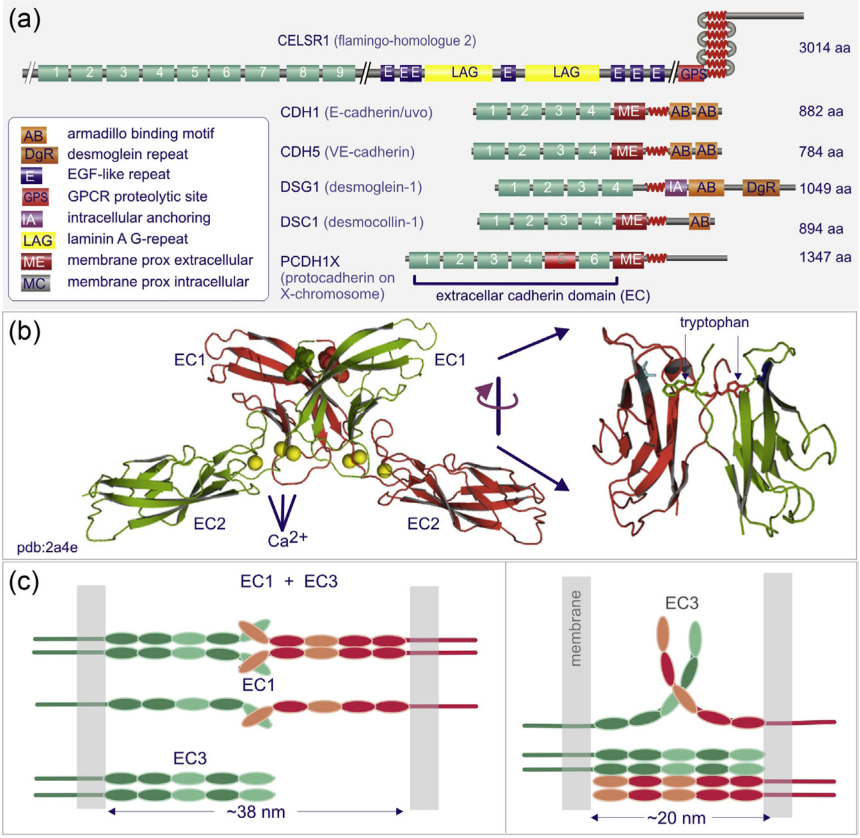


FIGURE 11-10 Cadherin domain structure. (a) Cadherins form a large family of adhesion molecules, characterized by extracellular cadherin (EC) domains and classified on the sequence of EC1. In many cases, the membrane proximal EC domain is only distantly related and better regarded as the membrane proximal extracellular domain (ME). Members of the major cadherin gene family (CDH) carry one or two armadillo-binding motifs. Armadillo-domain proteins, such as the catenins and desmoplakins, connect the adhesion molecules with the micro- (actin) and intermediate filaments, respectively (for details see Figure 11-11). PCDH1X is a member of the protocadherin family (PCDH). Representatives of the third family, cadherin-related genes (CDHR) are not shown. (b) A ribbon presentation of two cadherin repeats (EC1 and EC2 of cadherin-11) with Ca^{2+}-binding sites at the domain interface. The interaction between the two cadherins occurs through the N-terminal EC1 domain and is based on a domain-swap, in which tryptophan residues, (sphere or stick representation) play an important role. (c) Different modes of alignment of cadherins, employing both EC1 and EC3 domains. *Source: Information from Niessen et al. (2011).*

binding (plakoglobin). Finally, the family includes CELSR (flamingo-homolog) proteins, which typically have a much longer extracellular segment, a seven-pass membrane domain, and an unrelated cytoplasmic sequence compared to other cadherins (examples of the CDH subfamily are shown in Figure 11-10).

The protocadherin (PCDH) (http://www.genenames.org/cgi-bin/genefamilies/set/19) gene family encodes proteins with an extracellular segment comprising six or seven cadherin repeats with high sequence conservation within the family and weaker homology to the cadherin repeats of members of the major cadherin family. Further, protocadherins have a single transmembrane domain and a distinct, protocadherin-specific, cytoplasmic segment (one example shown in Figure 11-10). Protocadherins can be further subdivided into clustered and non-clustered protocadherins on the basis of particular genomic organizations. The third family, cadherin-related (CDHR) genes do not fit into the above two families as they are phylogenetically clearly separated. The encoded proteins comprise at least two typical, consecutive cadherin motifs, but often more than the typical members of the cadherin and protocadherin families. Their overall domain organization differs from the members of the major cadherin family and the protocadherin family, and includes different and unique cytoplasmic domains (not shown).

Go to the HUGO gene nomenclature committee (HGNC) webpage for detailed information about the Cadherin superfamily (gene family "CDH," covering all subfamilies). URL: http://www.genenames.org/cgi-bin/genefamilies/set/16.

Most, if not all, of what we know about the cadherins concerns the classical or type-I cadherins and in particular its archetype, E-cadherin (CDH1). Classical cadherins are transmembrane glycoproteins having five EC domains. Of these, EC1 to EC4 are very similar while EC5 is more distant and therefore referred to as the membrane proximal extracellular domain (ME). Interdomain stabilization is achieved through the binding of three calcium ions at the domain interfaces (Patel et al., 2006). Cadherins generally mediate homotypic cell–cell adhesion, acting as both receptor and ligand. Both types I and II cadherins exhibit adhesive interfaces confined to EC1 and EC3 domains (Patel et al., 2006). With respect to EC1, anchoring occurs through the insertion of a side chain (comprising tryptophan) into a complementary hydrophobic pocket in the partner molecule (panel (b), Figure 11-10). The strand exchange of cadherins exemplifies a more general domain-swapping strategy, which enables homophilic interactions between proteins having low affinity yet high specificity (Chen et al., 2005). Interactions between EC3 domains also occurs, and these play a role in clustering (*in cis*) and have also been observed between opposing cadherins (*in trans*) (panel (c), Figure 11-10) (Niessen et al., 2011).

Full functionality of the major cadherins (CDH) requires structural linkage to the cytoskeleton. To this end, they are organized as a "core complex." This includes β-catenin (CTNNB1) bound directly to the cytoplasmic segment of cadherin and α-catenin (CTNNA1) bound to the N-terminal region of β-catenin. Equivalents for desmosomal cadherins (desmocollin, desmoglein) are, respectively, plakoglobin (JUP) and desmoplakin (DSP). Cadherins of the adherens junction have a second catenin-binding site in the membrane proximal sequence and this interacts with δ-catenin (CTNND1). The interaction plays a role in the stabilization of the core complex by preventing uptake through endocytosis (Ishiyama et al., 2010). It also contributes to the recruitment of yet other proteins such as GTPases and tyrosine phosphatases (Lilien and Balsamo, 2005; Noren et al., 2000) (Figure 11-11). α-catenin connects the core complex to the actin cytoskeleton, either directly or indirectly, through interaction with actin binding proteins such as α-actinin (ACTN1), zona-occludens-1 (TJP1), vinculin (VCL), or formin (FMN1). Formin functions in actin polymerization, which means that cadherin-associated complexes contribute to the formation of the zonula adherens (subcortical contractile actin network). We return to this aspect later in the chapter (Vavylonis et al., 2006). All this suggests that the complex plays a role in the formation and maintenance of stable adhesions.

Catenins were discovered in a search for proteins that associate with uvomorulin. Three proteins of 102, 88, and 80 kDa were repeatedly co-immunoprecipitated and found to be present in mouse, chicken, and human cells. They were named catenin (α, β, and γ, respectively), from the Latin *catena*, meaning chain, since one major function was thought to be linking the adhesion molecule with the cytoskeleton (Ozawa et al., 1989).

In developmental processes, expression of each cadherin subclass is regulated both spatially and temporally and this is associated with individual morphogenic events. Thus, the tendency of cells to segregate or to aggregate correlates with the expression of particular cadherins. This is particularly apparent during gastrulation and the formation of the neural tube. In addition, regulation can also occur through activation, as occurs at the onset of compaction (Figure 11-9) or in the process of axon guidance where local changes in cadherin adhesion steer the growth cone. Axon guidance cues, like Neurocan and Slit, rapidly inactivate cadherin through the phosphorylation of its intracellular partner β-catenin (Li et al., 2000; Rhee et al., 2002). By eliminating cadherin-mediated traction, these axon-guiding cues allow for growth-cone extension. Many of the cadherins are implicated in

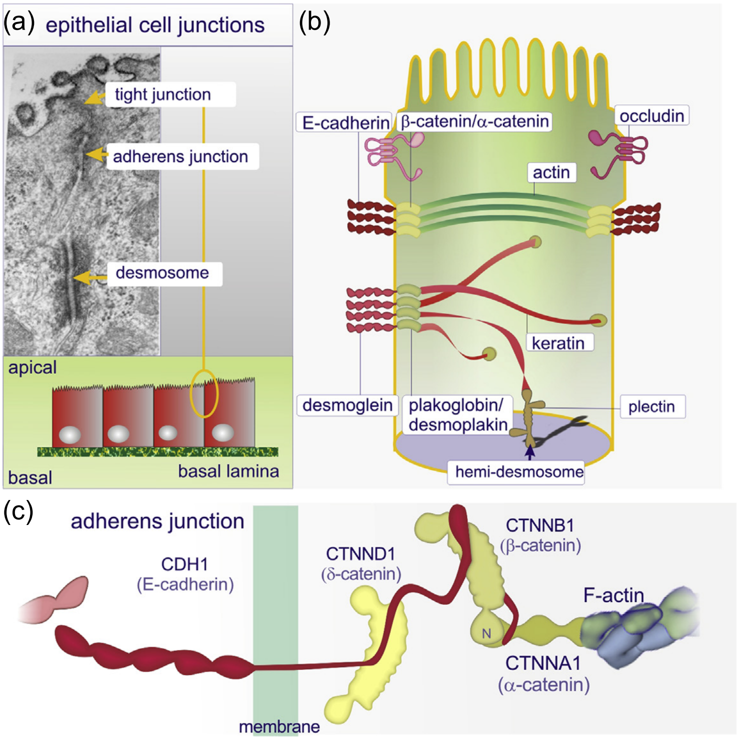


FIGURE 11-11 Epithelial junctions. (a) Epithelial cells are firmly attached to each other by tight junctions, adherens junctions, and desmosomes and they attach to the basement membrane with hemi-desmosomes. All of these play an important role in maintaining both cellular and tissue integrity and warranting selective exchange of solutes between anatomical compartments. (b) Adherens junctions are formed by homophilic interactions between members of the cadherin family and they are connected to the cellular adhesion belt formed of contractile-actin filaments (zonula adherens). α- and β-catenin make the connection between the two. Desmosomes are formed by homophilic interactions of desmogleins (or desmocollins), linked to intermediate filaments (keratin in the case of epithelial cells). Here, plakoglobin and desmoplakin make the connection between the two. At the basal membrane these filaments form a hemi-desmosome, interacting with integrins via plectin. (c) The tight association between cadherin, β-catenin, α-catenin, and filamentous actin illustrates the physical stress-resisting quality of cell junctions.

long-term potentiation, memory formation, and spatial learning, most likely through their capacity to form synaptic contacts and thus spatially organize the brain cortical layer (Curran & D'Arcangelo, 1998). By forming cell–cell contacts, the cadherins appear to act as tumor suppressors and when these fail, as in metastasizing epithelial tumors, there is a loss of basolateral localization (more in Chapter 12, "Wnt Signaling and the Regulation of Cell Adhesion and Differentiation").

Review about cadherins with emphasis on its role in tissue organization during development (Niessen et al., 2011).

Cadherins at a glance: Saito et al. (2012).

Web resource

Kyoto encyclopedia of genes and genomes, pathway map 04520 "adherens junction," URL: www.genome.jp/kegg-bin/show_pathway?map04520.

SELECTINS

Selectins present on the surface of white blood cells, platelets, and also the endothelial cells that line blood vessels, form a small family of adhesion molecules (the selectin/LECAM family, leukocyte–endothelial cell-adhesion molecules). They are categorized among the Cell Differentiation (CD) gene family in the HUGO-gene nomenclature classification. They mediate the initial low-affinity adhesion sites for lymphocytes and for leukocytes such as neutrophils. This prepares the way for cell migration out of the vasculature and into the lymph nodes and tissues (immunosurveillance). Animal selectins (see textbox) were discovered in studies dealing with lymphocyte recirculation, a process comprising "homing," going from blood to lymphoid organs, and then back into the blood (Gowans and Knight, 1964). Lymphocytes can be displaced from lymph nodes by monosaccharides (L-fucose and D-mannose) and by the polysaccharide fucoidin (rich in L-fucose) giving an indication that carbohydrate residues may be involved in their homing (Stoolman and Rosen, 1983). This was confirmed by the finding of a lymph node-specific homing receptor that possesses a C-type lectin domain (C for Ca^{2+} binding) (Lasky et al., 1989). Various selectins, mediating intercellular interactions, have since been identified in vascular endothelial cells (E), platelets (P), and leukocytes (L) (gene names SELE, SELP, and SELL) (panel (a), Figure 11-12). The N-terminal C-type lectin domain interacts with oligosaccharide structures, usually presented on protein scaffolds. Sialyl lewis(x) (sLex) and related structures, containing fucose, galactose, N-acetyl glucosamine, and sialic acid, are recognition determinants for this type of lectin domains (panel (b), Figure 11-12). Selectins also contain a single EGF-like domain and a series of Sushi repeats (also referred to as complement regulatory proteins, CRP) (Figure 11–12). The EGF-like domain plays a mechanistic role in the catch-and-slip quality of these adhesion molecules (see below).

Selectin: Homologous to the Ca^{2+}-dependent (C-type) lectin. Lectin is derived from the word select, and originally applied to plant proteins that bind to specific carbohydrate residues present on nitrogen fixing bacteria. Lectins have been used in cell biological work because they bind to specific glycosidic residues present in the Golgi system and on cell surfaces. Only later it was found that animal cells also possess similar proteins, generally on the surfaces of endothelial and myeloid cells.

L-selectin is responsible for lymphocyte homing and is expressed on all circulating leukocytes except for a subpopulation of memory cells. It recognizes CD34 (a heavily glycosylated mucin) on endothelial cells. Other ligands are SELPLG, addressin (MADCAM), and endoglycan (PODXL2) (Rosen, 2004). E- and P-selectins are inducibly expressed on endothelial

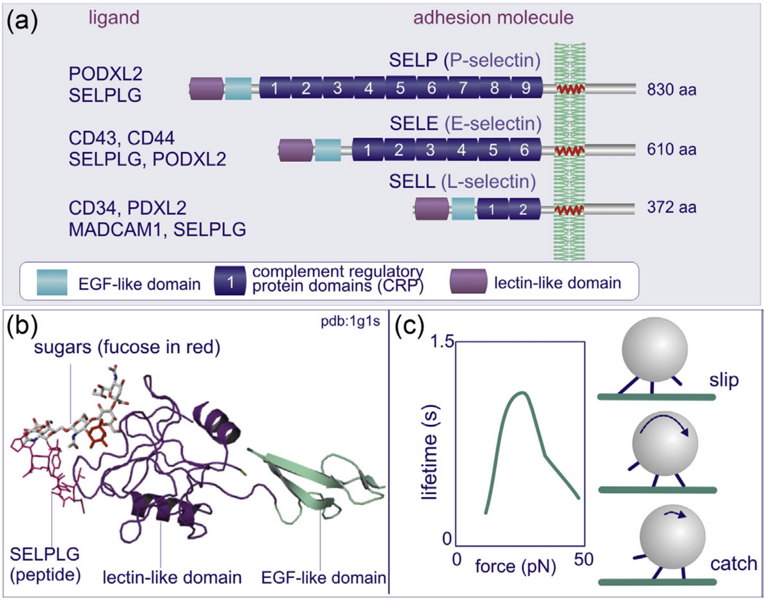


FIGURE 11–12 Domain structure and ligands of selectins. (a) The selectins are characterized by the N-terminal lectin-homology domain and a variable number of complement regulatory protein domains (CRPs). Through their lectin domain, selectins interact with sugar residues present in cell surface glycoproteins and glycolipids (ligands). (b) Ribbon presentation of the lectin-like domain of E-selectin bound to PSGL-1. Sugars in stick representation, fucose is red. (c) Lifetime of the bonding of P-selectin to SELPLG increases with force, then decreases. This may explain the rolling of white blood cells (that express P-selectin) on the vascular endothelium (that expresses SELPLG) under shear stress (blood flow). *Source: Image adapted from Yago et al. (2004) and Marshall et al. (2003).*

cells and mediate the recruitment of eosinophils, neutrophils, monocytes, natural killer cells, and effector lymphocytes involved in many inflammatory processes. E- and P-selectins recognize SELPLG on leukocytes, other ligands are endoglycan (PODXL2), CD43, and CD44 (hyaluronan receptor).

Selectins have the remarkable property that allows their affinity for ligands to be modulated by the imposition of shear forces (Alon et al., 1998).The interaction between L-selectin and SELPLG shows a biphasic relationship by which low shear forces decrease off-rates, the bond lifetime being prolonged ("catch bonds"), while higher shear forces increase off-rates ("slip bonds"). It appears, although the molecular details remain uncertain, that shearing deforms the interacting molecules such that they lock more tightly (Marshall et al., 2003).This behavior, catch-and-slip, might explain the rolling of leukocytes on the vascular endothelial layer under flow conditions (panel (c), Figure 11-12) (Ivetic and Ridley, 2004).

Little is known about the role of the selectins or the selectin-binding molecules in signal transduction. Several binding partners including α-actinin, calmodulin, and members of the ezrin/radixin/moesin (ERM) family of cytoskeletal proteins attach to the short cytoplasmic tail (17–35 residues) of selectins.

CARTILAGE LINK PROTEINS

The glycosaminoglycan hyaluronan, a high-molecular-weight polysaccharide, is present in the tissue matrix and body fluids of all vertebrates. It plays a fundamental role in regulating cell migration and differentiation (Laurent and Fraser, 1992). The majority of hyaluronan-binding proteins carry a conserved Link domain (approximately 90 residues) that has a three-dimensional structure resembling the sugar-binding domain of E-selectin (Figure 11-13). They can be further subdivided into two groups, those that are true cellular adhesion molecules such as CD44 and LYVE1. In the HUGO gene-name classification they are categorized among different families, the "CD," "blood group antigens," and "proteoglycans" (Aruffo et al., 1990; Banerji et al., 1999). The other group concerns components of the extracellular matrix such as cartilage-link protein itself, aggrecan (ACAN), brevican (BCAN), Neurocan (NCAN), TNFAIP6, and versican (VCAN). These are categorized among the "hyalectans" (Day, 1999).The hyalectans play an important role in the architecture of the extracellular matrix, bringing together the pressure-resistant qualities of glycosaminoglycans (very long complex-sugar chains) and the tension-resistant qualities of the extracellular matrix proteins. Hyalectans combined with hyaluronan and collagen make up the essential quality of cartilage in articulated joints.

Glycosaminoglycans and the extracellular matrix proteins, in particular collagen, share with wood and reinforced concrete the valuable qualities of resistance to the forces of both tension and compression. These are two important requirements for the construction of not only organisms but also buildings.

Sialyl Lewis are sialylated sugars expressed on blood cells that serve as antigens in blood group typing as developed by Lewis.

CD44, originally discovered as the homing receptor of lymphocytes, is the best studied of the cartilage link proteins. It is required when lymphocytes bind to high endothelial venules and exit the circulation, seeking lymph nodes (Goldstein et al., 1989). In a manner reminiscent of the integrins expressed on circulating blood cells, CD44 is functionally inactive. It binds hyaluronan only after T-cell receptor triggering or exposure of the lymphocytes to inflammatory cytokines such as TNF-α and INF-γ. In this way, selected lymphocytes are directed to inflammatory sites by binding to hyaluronan present on the surface of the vascular endothelial cells.

CD44 is a single-membrane spanning protein encoded by 19 exons of which nine in the extracellular domain (v2–v10) are variably spliced (Figure 11-13). Because of the extensive glycosylation and the sequence variability, the molecular mass of CD44 can vary widely, anywhere between 85 and 200 kDa. The link-domain is implicated in the binding of hyaluronan but the splicing extends the range of binding capacities. For instance, the inclusion of exon v3 allows the attachment of heparan or chondroitin sulfate, enabling it to interact with fibroblast growth factor-4 or -8 (FGF-4 or -8), with hepatocyte growth factor/scatter factor (HGF) and E-selectin. Other potential ligands are osteopontin (SPP1), fibronectin (FN1), and ankyrin (ANK1). Homophilic intercellular interactions with CD44 are also reported.

The splice variants stimulated great interest when it appeared that CD44v4–7 might determine metastasis in rat pancreatic tumor cells. In short, it seemed at first to have the quality of a metastasis gene product (Gunthert et al., 1991). More than this, a monoclonal antibody raised against v6 prevents metastasis. Unfortunately, however, although human tumors often express splice variants of CD44 and in certain instances this predicates poor prognosis, there seem to be no functional implications. The signal transduction pathway emanating from CD44 remains uncertain. A number of membrane proteins, including ezrin (EZR), radixin (RDX), and moesin (MSN), that associate with it, belong to the group of erythrocyte band 4.1-related proteins. These are proposed to function as links between the membrane and the cytoskeleton (Tsukita et al., 1994), but it remains to be shown that they actually relay signals from CD44 into the cell. Another point of interest

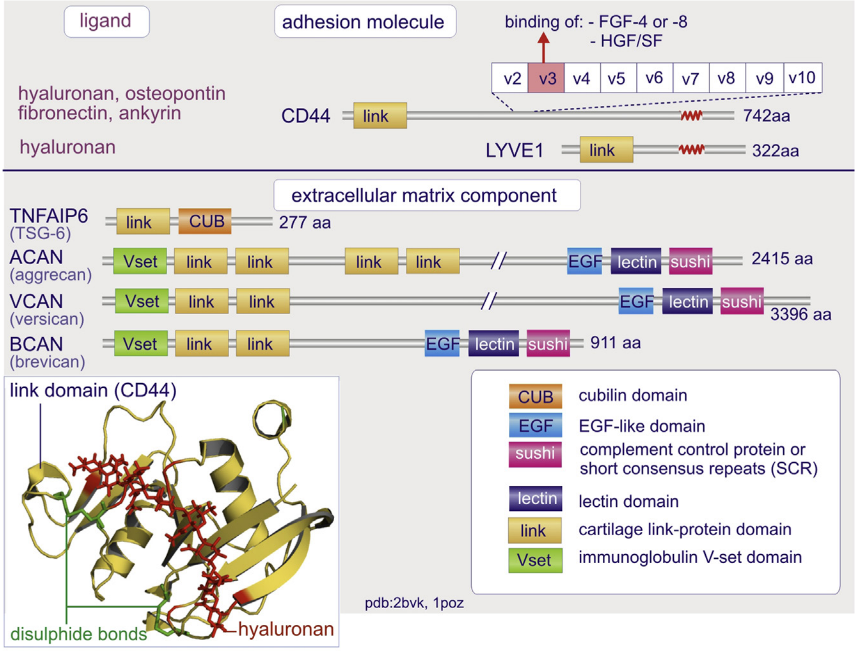


FIGURE 11-13 Domain structure of the cartilage link proteins. Cartilage link proteins come in two forms, those that are transmembrane proteins and act as receptors and those that are extracellular matrix proteins and play an important role in connecting glycosaminoglycans with proteins. They are characterized by the link domains that bind hyaluronan. CD44 has the capacity to form many splice variants (inserted variable exons v2 to v10), which provide new binding properties independent of the link domain. The inset shows a ribbon presentation of the link domain, stabilized by two disulfide bonds, connecting CD44 to a short stretch of hyaluronan.

is of course, the capacity of CD44v3 to bind growth factors of the FGF and HGF families. This may mean that it is involved in the recruitment of the respective receptors, thereby mediating intracellular signaling. In the following sections, signaling to and from CD44 will not be further discussed.

INTEGRINS, CELL SURVIVAL, AND CELL PROLIFERATION

Inside-out signaling and the formation of integrin–adhesion complexes

Cells in culture organize their contacts with the extracellular matrix in the form of integrin clusters (see upper panel of Figure 11-15). These are composed of a number of proteins, some having structural, and others

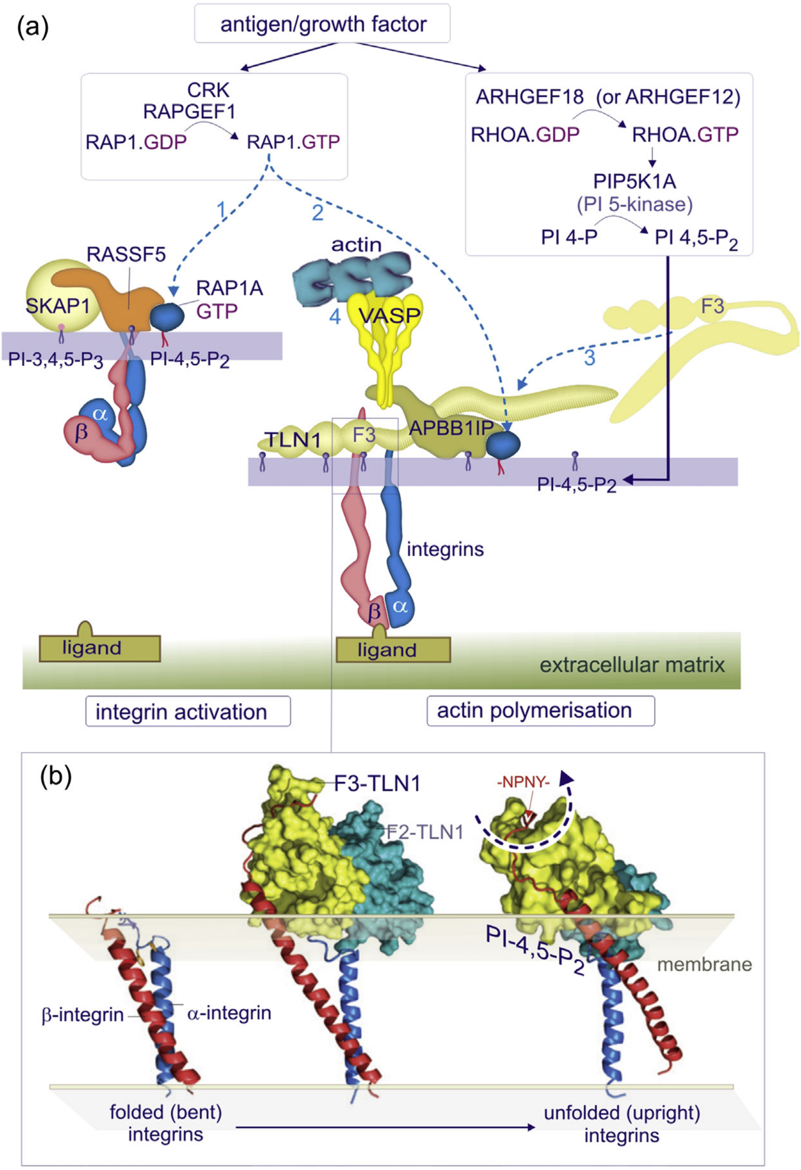


FIGURE 11-14 Receptor-mediated activation of integrins. (a) Tyrosine phosphorylated growth factor or T-cell antigen receptors bind the adaptor CRK that links to the guanine nucleotide exchange factor RAPGEF1 and activates the monomeric GTPase RAP1. This addresses two effectors, Rap-ligand (gene name RASSF5) and Riam (gene name APBB1IP). RASSF5, when recruited to the membrane through SKAP1, binds the cytosolic domain of the α-subunits of integrins and is implicated in their activation. APBB1IP is recruited to the membrane by inositol lipids and binds both talin-1 (TLN1) and VASP. The latter promotes actin filament elongation and contributes to the elaboration of an actin cytoskeleton associated with focal adhesion sites. In parallel, receptors also cause activation of the RHOA guanine exchange factors ARHGEF18 or ARHGEF12. These activate PI5-kinase with production of PI4,5-P2 necessary for membrane attachment of APBB1IP. (b) A composite representation of αβ integrin subunits bound to the F3 region of the

signaling roles. Formation of such focal adhesion sites involves activation of integrins, their binding to a component of the extracellular matrix (fibronectin, vitronectin, laminin, or collagen), the formation of actin filaments, and their attachment to these newly formed filaments. This process also requires the monomeric GTPases RAP1 and RHOA.

RAP1 contributes to integrin activation and more generally, it is a key regulator for the adhesion of blood-borne cells and platelets (Reedquist et al., 2000; Franke et al., 2000). Here, it is activated by the guanine nucleotide exchange factor RAPGEF1 (Tanaka et al., 1994) recruited to tyrosine phosphorylated receptor tyrosine kinases (PDGFR or EGFR) via the SH2/SH3-adaptor protein CRK (see textbox below). Fibroblasts deficient in RAPGEF1 display impaired adhesion and accelerated migration (Ohba et al., 2001). Loading RAP1 with GTP allows an interaction with two effectors that play a role in integrin activation. One of these is RAP Ligand (gene name RASSF5) that associates with the membrane-bound SKAP1 and together they interact with the α-chain of integrins (Katagiri et al., 2003; Raab et al., 2010). Mice lacking RASSF5 show an impaired transendothelial migration because their integrins fail to attach firmly to the endothelial cells (further discussed in Chapter 14, "Chemokines and Traffic of White Blood Cells"). The other effector is Riam (gene name APBB1IP) that, in lymphocytes, localizes RAP1-GTP to adhesion structures (Lafuente et al., 2004) through its interaction with phosphatidyl 4,5-phosphate (Figure 11-14). It binds both TLN1 and VASP. VASP facilitates elongation of actin filaments that attach to the focal adhesion contact.

Kyoto encyclopedia of genes and genomes (KEGG), consult the "focal adhesion" pathway entry hsa04510 for proteins associated with focal adhesion sites. URL: http://www.kegg.jp/kegg-bin/show_pathway?hsa04510.

The CRK adaptor protein was initially discovered as a virus-encoded oncogene. Here is a good example of how viruses mess about with genes resulting in chimeric proteins. In this case, viral Gag sequences are fused to the cellular protein CRK, an SH2 and SH3 domain-containing adaptor. The human genome contains two homologs CRK and CRKL. Of these, CRKL is implicated in the pathogenesis of chronic myelogenous leukemia.

◀ FERM domain of TLN1. The model is obtained by combining the NMR structure of two integrin transmembrane domains (on the left) with the structure of the F2–F3 domains of TLN1 bound to a β1 integrin tail (in which the peptide sequence -NPNY- takes part). The formation of favorable electrostatic interactions between F2 and F3 of TLN1 and the membrane, in part caused by the production of negatively charged PI4,5-P2, brings about a rotation of the TLN1-β-integrin tail complex (right structure) thus increasing the tilt of the β-integrin transmembrane helix. This leads to separation of the α-subunit and β-subunit transmembrane domains and hence to the unfolding of the integrin extracellular segment. *Source: Image adapted from Calderwood et al. (2013).*

RHOA mediates its effect in the assembly process through the activation of phosphatidylinositol 5-kinase, generating PI4,5-P2 (Figures 11-14 and 11-15). As a result both APBB1IP (RIAM) and vinculin (VCL) are able to attach to the plasma membrane. In the case of vinculin, this unmasks

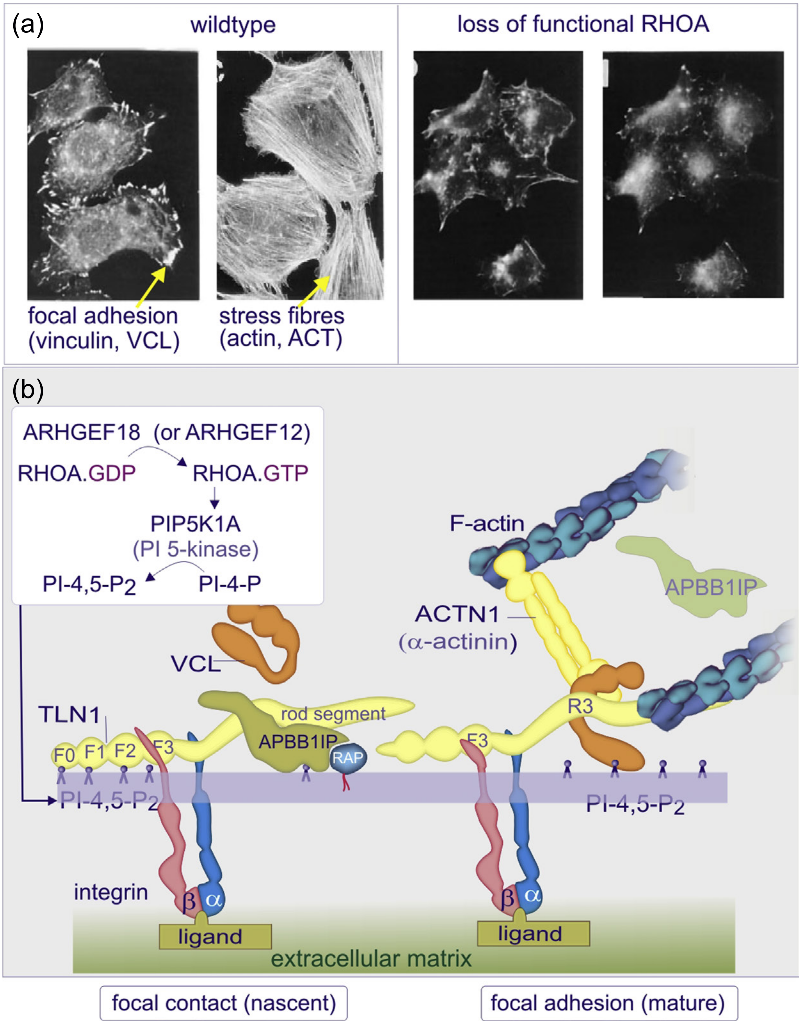


FIGURE 11-15 RhoA-mediated formation of focal contact sites. (a) The role of RHOA in focal contact formation is nicely illustrated in the micrographs of cells stained with fluorescent phalloidin (specifically binds to filamentous actin) or with antibodies directed against vinculin. In the presence of matrix proteins and serum, the fibroblasts spread out, forming focal adhesion sites and stress fibers (left panel). However, when a nonfunctional RHOA is introduced, strong focal contacts are not formed and actin stress fibers are absent. *(Adapted from Clark et al. (1998).)* (b) Growth factors play an essential role in the formation of focal adhesion sites through activation of the Rho nucleotide exchange factors ARHGEF18 or ARHGEF12. GTP-bound RHOA promotes formation of PI4,5-P2 through activation of PI5-kinase. Vinculin (VCL) attaches to the PI4,5-P2, causing it to open up so that it attaches to both talin-1 (TLN1) and α-actinin (ACTN1), making a firm link between the integrins and the actin cytoskeleton.

cryptic binding sites that allow it to form a cross bridge between talin (which is linked to the β-subunit of integrins) and to α-actinin (which is linked to filamentous actin) (Gilmore and Burridge, 1996) (Figure 11-15). Binding of vinculin to talin-1 must occur at a later stage because it competes with the binding of APBB1IP. Connecting vinculin with TLN1 plays a role in the maturation, not in the initiation, of the focal-adhesion site. A solid structure, integrating extracellular matrix and actin cytoskeleton is thus formed.

Outside-in signaling from integrin–adhesion complexes

Anchorage-dependent growth and survival

An essential requirement for the proliferation of tissue cells driven by growth factors is their attachment to a suitable surface. The fact that cells must adhere to substrate provides a likely molecular explanation for the anchorage-dependent growth of cells that form part of a solid tissue. Adherence-dependent signaling targets have much in common with growth factor-signaling targets (described in Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases" and in Chapter 16, "Signaling through the Insulin Receptor") and they are seemingly redundant. Signaling targets are evident in various stages of the RAS–MAP-kinase pathway; upstream of RAS, between RAS and RAF, or between RAF and MEK (MAPK2). Other targets are the JNK pathway, STAT-mediated regulation of transcription, and the AKT pathway. For all of these, adherence and growth factor signaling act in synergy, one amplifies the other and this is necessary to engage the cell in a round of proliferation. The survival of endothelial and epithelial cells also depends critically upon the contacts they make with the extracellular matrix (and with each other). Without contact, they die through the controlled process of apoptosis, which in this particular context is referred to as "anoikis" (meaning homeless) (Frisch and Francis, 1994). Cells have an intrinsic drive to self-destruct, but are prevented from doing so by signals emanating from specific rescue pathways. One such signal (outside-in) follows from the attachment of the integrins to the extracellular matrix. This mechanism may have evolved to protect the organism against stray cells colonizing inappropriate locations (metastasis). The section below illustrates integrin-dependent signaling pathways that control cell survival and early events in the cell cycle (G1 phase).

The formation of an integrin signaling complex

The focal adhesion complex forms the site of attachment for the tyrosine kinase PTK2 (initially name Focal Adhesion Kinase or FAK) (Figure 11-16). Recruitment to the nascent focal contact occurs through binding of the focal

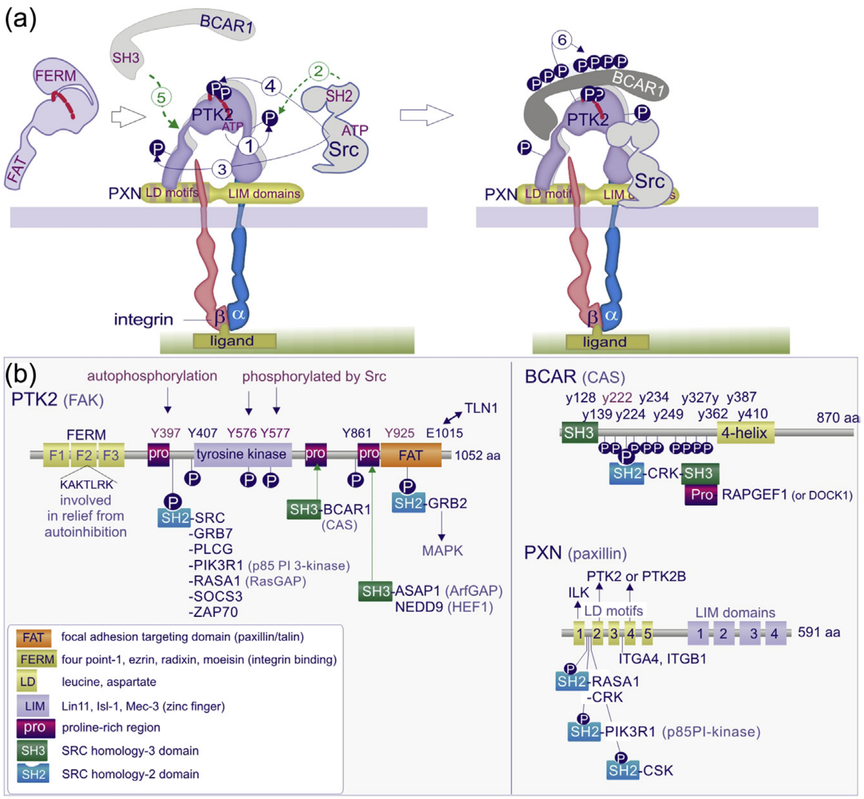


FIGURE 11-16 A two-step process to create an integrin signaling complex. (a) With the structural components of the focal-adhesion site assembled, the focal-adhesion kinase PTK2 associates with one of the LD motifs of paxillin (PXN). Autophosphorylation of PTK2 on Y397 generates a docking site for the SH2 domain of the SRC tyrosine-protein kinase. This in turn phosphorylates two tyrosines in the activation segment (Y576 and 577) and a tyrosine in the FAT domain, Y925. The phosphorylated PTK2 recruits BCAR1, through binding of its SH3 domain to a proline-rich sequence at the interface between the FAT and kinase domains. BCAR1 is phosphorylated by SRC and PTK2 at multiple sites. An integrin-signaling complex is formed that acts in a manner similar to growth factor-receptor signaling complexes; that is, attachment of adaptors and effectors and tyrosine phosphorylation of substrate (see also Figure 11-18). (b) Illustration of the domain architectures and essential phosphorylation sites of PTK2, BCAR, and PXN.

adhesion motif (FAT) to one of the LD motifs of paxillin (PXN) (Hoellerer et al., 2003; Lawson et al., 2012). Paxillin itself is bound to the intracellular segment of β-integrins. Attachment, resulting in autophosphorylation on Y397, enables PTK2 to act as a docking site for the tyrosine protein kinase SRC (and also other members of the SRC family of kinases such as FYN and YES)

(Prutzman et al., 2004). SRC phosphorylates two tyrosine residues in the activation segment (Y576, Y577) converting PTK2 in an active kinase. Phosphorylation of other tyrosines follows. Besides binding SH2 domains, PTK2 also possesses two proline-rich regions one of which interacts with the SH3 domains of BCAR1 (also known as CAS) (Lim et al., 2004) (Figure 11-16). BCAR1, endowed with many tyrosine phosphorylation sites that are phosphorylated by SRC and PTK2, acts as a phosphotyrosine-docking protein attracting numerous SH2-containing effectors and adaptors. A lack of BCAR1 prevents integrin-mediated PTK2 signaling (Iwahara et al., 2004). The role of PTK2 is not limited to the initiation of focal adhesion complexes. It also plays a role in the recruitment of talin-1 and may at an even later stage be involved in switching-off the integrins, as occurs in the process of cell migration (Lawson et al., 2012).

A review about PTK2: (Hall et al., 2011).

The name paxillin, derived from the Latin *paxillus*, a stake or peg, is consistent with its proposed function in linking actin filaments to integrin-rich cell adhesion sites. For a review about paxillin see Brown and Turner (2004).

Protection against apoptosis in circulating cells, such as leukocytes, cannot occur through adhesion contacts and imposes a different mechanism. For sure, some white blood cells such as neutrophils are very short lived (just a few hours in the blood), but others such as the lymphocytes responsible for immunological memory, survive for years. Whether memory cells need to be continually reminded to survive by occasional encounters with other cells or an extracellular matrix remains to be established. Interestingly, lymphocytes employ an alternative pathway to activate AKT, which involves the GTPase RAC1 (Genot et al., 2000).

The importance of PTK2 is underlined by the finding that cells expressing a constitutively active form, survive in suspension even though they are effectively “homeless” (Frisch et al., 1996). Here, PTK2 is active regardless of the failure to make contact with an extracellular matrix.

BCAR1 (also known as Crk-associated substrate) belongs to the CAS-family of proteins. Other members are NEDD9, CASS4, and EFS. They are SH3-containing docking proteins with multiple tyrosine phosphorylation sites that interact with SH2 domains of adaptors (such as CRK) and tyrosine kinases (FYN, SRC, PTK2, LYN), or tyrosine phosphatases (TPTN1, PTPN12). In humans, BCAR1 was identified as a protein whose overexpression confers resistance to antiestrogen treatment (tamoxifen) of breast cancer (BCAR1, hence its name) (Brinkman et al., 2000). BCAR1 is an important mediator of SRC-mediated cell transformation. BCAR1 phosphorylation correlates with inhibition of expression of FHL1 and this loss serves as a marker for anchorage-independent cell growth (oncogenic transformation) (Shen et al., 2006).

Web resource

For a complete list of (some 150) proteins involved in focal adhesion complexes we suggest the following website: http://adhesome.org (Zaidel-Bar et al., 2007).

PTK2-mediated activation of AKT and cell survival

Among the proteins that attach to tyrosine-phosphorylated PTK2 is the p85-regulatory subunit (PIK3R1) of PI3 kinase (PIK3CA) (Figure 11-17). This generates 3-phosphorylated inositol lipids with the subsequent membrane recruitment of PKD1 and AKT leading to activation of AKT (a serine/threonine protein kinase) (see Chapter 16, “Signaling through the Insulin Receptor”). AKT effects a number of phosphorylations that promote rescue by at least four different mechanisms (Figure 11-17) (King et al., 1997; Khwaja et al., 1997; Downward, 2004). The first is by direct phosphorylation and inactivation of components of the apoptotic machinery, including BAD, CASP9, and XIAP (Downward, 1998). Phosphorylation of CASP9 prevents its proteolytic activation while phosphorylation of BAD shifts its location from the mitochondrial membranes to the cytosol due to sequestration by 14-3-3 proteins. Since the phosphorylation site of human CASP9 is not conserved in mouse and rat it seems unlikely that this is a major control mechanism.

AKT acts to control transcription of a number of genes that regulate apoptosis by phosphorylating the three members of the FOXO subfamily of Forkhead transcription factors (FOXO1, FOXO3, and FOXO4) causing cytosol retention, again by 14-3-3 proteins. In this way, they are prevented from activating genes critical for induction of factors that promote cell death such as FASLG (Brunet et al., 1999), TNFSF10 (TRAIL)

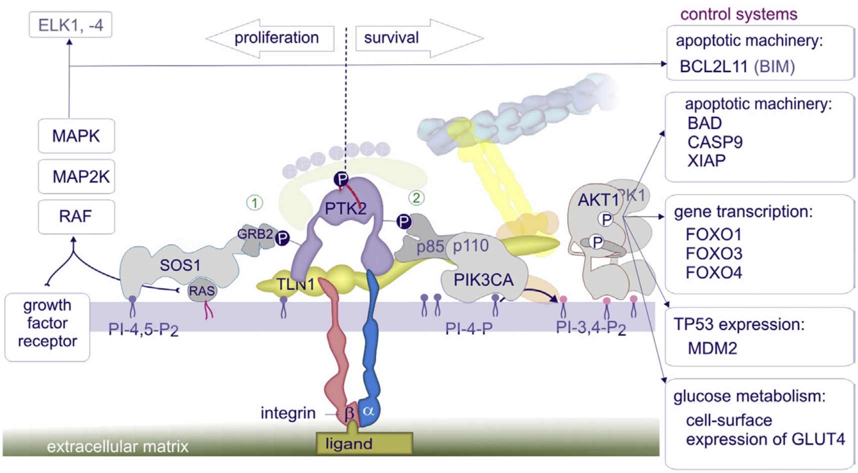


FIGURE 11-17 Adhesion-mediated survival and proliferation. The focal adhesion site promotes cell proliferation signals through activation of RAS. The SRC phosphorylated Y925 acts as a binding site for the SH2 domain of GRB2. This interaction recruits the RAS guanine nucleotide exchange factor SOS1 to the membrane, leading to activation of RAS. RAS-GTP initiates the activation of the RAF–ERK pathway, necessary for initiation of the cell cycle. The focal adhesion site promotes cell survival signals through activation of the serine/threonine AKT. The phosphorylated tyrosine residue Y397 of focal adhesion kinase (PTK2) provides a binding site for the SH2 domain of the regulatory subunit (PIK3R1 or p85) of the phosphatidyl inositol 3-kinase (PIK3CA). Subsequent production of PI3,4,5-P3 provides a binding site for the PH domain of both PDK1 and AKT. After its activation by PDK1, AKT phosphorylates a large number of proteins that directly or indirectly deal with cell death.

(Modur et al., 2002), TRADD, and the pro-apoptotic protein BCL2L11 (Bim) (Dijkers et al., 2000). That this constitutes an essential survival mechanism is indicated by the finding that overexpression of FOXO3 triggers a program of cell death in the interleukin-3 dependent hematopoietic cell line Ba/F3 (Dijkers et al., 2002).The death process is identical to that which occurs after withdrawal of the cytokine interleukin-3 (normally essential for these cells' survival) (Figure 11-17).

Another substrate of AKT, MDM2, is a component of an E3 ubiquitin ligase complex (see "Two classes of E3-ubiquitin ligases," Chapter 13, "Activation off the Innate Immune System," Figure 13-14) that ubiquitylates the tumor-suppressor protein TP53, preparing it for destruction by the proteasome. MDM2-assisted destruction of TP53 occurs in cells that have undergone DNA damage ("genotoxic stress") but which aspire to resist apoptosis. Phosphorylation of MDM2 causes its nuclear localization and renders TP53 ubiquitylation more efficient (Zhou et al., 2001). As with CASP9, this mechanism of protection by AKT is operational in human cells, although the phosphorylation sites are not conserved throughout mammalian species.

The fourth mechanism involves AKT control of carbohydrate metabolism. When cells are deprived of growth factors, nutrient uptake is severely reduced and they become quiescent. This may reach such a point that they fail to maintain their size and this inevitably results in a loss of viability (Plas et al., 2002). The apoptotic process that ensues is due to the enhanced permeability of the mitochondrial outer membrane to cytochrome c (CYCS). In cells starved of glucose, this is signaled by detachment of hexokinase from the mitochondrial outer membrane. Beyond its widely recognized role in phosphorylating glucose, hexokinase may also be involved in the linkage between the mitochondrial outer membrane pores and the inner membrane adenine nucleotide transporter, ensuring pore integrity and transport selectivity. Sustained activation of AKT prevents apoptosis of cells even in the absence of growth factors by maintaining the cell surface expression of the glucose transporter GLUT4 (see Chapter 16, "Signaling through the Insulin Receptor"). As a consequence, the activity and the mitochondrial association of hexokinase are maintained and leakage of cytochrome *c* is prevented (Majewski et al., 2004).

Finally, the action of AKT is certainly not limited to these survival processes. It also protects cyclin D1 (CCND1) against degradation and stimulates protein synthesis through activation of the kinase MTOR (discussed in Chapter 16, "Signaling through the Insulin Receptor").

We note that ERK, normally involved in driving cell proliferation (see below) is also involved in protection against apoptosis. It prevents expression of BCL2L11 (Bim) a so-called BH3-only protein that activates BAX on the membrane of the mitochondrion and creates the mitochondrial outer-membrane permeabilization pore (Mailleux et al., 2007; Reginato et al., 2003).

Cell proliferation and PTK2-mediated reinforcement of the RAS–ERK pathway

Clustered integrins bind PTK2 that undergoes autophosphorylation and then recruits SRC to cause further phosphorylation and the formation of an activated tyrosine-protein kinase complex. With most integrins, the phosphorylated PTK2 binds the adaptor complex GRB2/SOS1 and then activates the RAS–ERK pathway (Figure 11-17) (Mitra et al., 2006). With other integrins (α1β1, α5β1, and αVβ3), activation of ERK pathway requires the palmitoylated-kinase FYN that links to the α-subunit and, through SH3 linkage to SHC1. SHC1 is then phosphorylated and this leads to recruitment of the GRB2/SOS1 complex (Wary et al., 1998). This additional RAS–ERK signal is important for a sustained activation of ERK. If EGF or PDGF are provided to suspended fibroblasts, the activation of the ERK pathway is merely transient; there is no increase in the expression of cyclin D1 and expression of the cell-cycle inhibitors $p21^{CIP/WAF}$ (gene name CDKN1A) and $p27^{KIP}$ (gene name CDKN1B) is fully maintained. The cells fail to proliferate and, sooner or later, they undergo apoptotic death (Assoian, 1997).

An alternative route for the stimulation of ERK is through phosphorylation of BCAR1 by PTK2/SRC. Tyrosine-phosphorylated BCAR1 preferentially (not exclusively) binds the SH2/SH3 adaptor-protein CRK, which is either linked to the RAP1 guanine exchange factor RAPGEF1 or to the RAC1 exchange factor DOCK1 (Hasegawa et al., 2006; Kiyokawa et al., 1998). This results in activation of both RAP1 and RAC, leading to activation of BRAF, with prolonged activation of ERK, and leading to activation of JNK1 (Figure 11-18) (Bottazzi et al., 1999; Lenormand et al., 1993). The sustained signal ensures progression from G_0 to G_1 and entry into the cell cycle (Aplin et al., 2001).

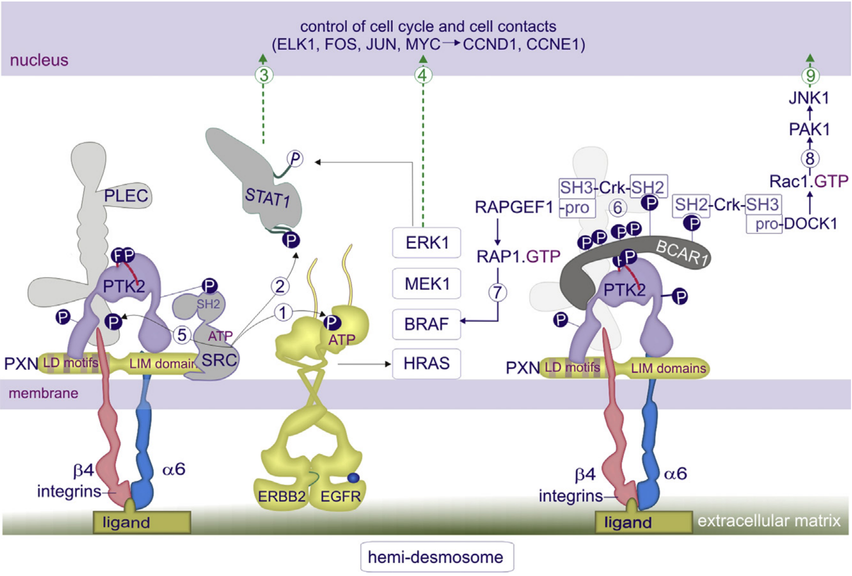


FIGURE 11-18 PTK2-mediated signaling. In epithelial cells, integrin α6β4, attached to the extracellular matrix forms a hemi-desmosome, a specialized adhesion complex. These are linked to intermediate filaments via plectin (PLEC). (1) EGFR/ERBB2 receptors are recruited, leading to phosphorylation of ERBB2 by SRC bound to PTK2. This promotes the activity of the ERBB2 kinase and enhances the growth factor-receptor signaling output. (2) SRC also phosphorylates STAT1, this signal being enforced by a second (serine) phosphorylation by ERK2. ERK2 and STAT1 cooperate (3 and 4) in the regulation of cell–cell contacts and the cell cycle (cyclin-D, MYC JUN, FOS, and more). In the case of breast tumor cells, this pathway promotes cellular invasion. (5) Finally, the β4-integrin (ITGB4) subunit is also a target of SRC and this may affect its interaction with plectin. The ghosts decorating BCAR1 indicate tyrosine phosphorylation sites. (6) The phosphotyrosines of BCAR1 bind the SH2 domain of CRK. The proline-rich region (pro) of RAPGEF1 and DOCK1 (guanine exchange factors) bind the SH3 domain of CRK. Their recruitment to the focal adhesion complex causes the activation of RAP1 and RAC1. (7) Rab1 signals to BRAF, which then phosphorylates MEK1, thus, enforcing the growth factor receptor-stimulated RAS–ERK pathway, whereas (8) Rac1 stimulates PAK1, which signals to JNK1. (9) JNK translocates to the nucleus and also stimulates expression of genes that initiate progression into the G1 phase of the cell cycle (cyclinD, MYC, etc.).

PTK2-mediated regulation of cell-cycle inhibitors

Focal adhesion sites also regulate the expression of the cell cycle inhibitor p27^{KIP} (CDKN1B) that plays a dual role in cell-cycle regulation. At low levels it promotes progression through G1 because it stabilizes the complexes of cyclin-D1 (CCND1) with CDK4 or CDK6 and it enhances nuclear retention, resulting in a more effective phosphorylation of the retinoblastoma protein. At high concentrations it blocks proliferation by inhibiting the activity of the cyclin complexes. Replacement of PTK2 with a kinase-dead mutant blocks DNA synthesis due to growth factors and this coincides with sustained elevated levels of p27^{KIP}.

PTK2 effectively controls the expression level of p27^{KIP} by stimulating its destruction. More directly, it acts to induce SKP2, the receptor component of one of the different SCF E3-ubiquitylation complexes. The elevated levels of SKP2 augment the assembly of SCF^{Skp2} complexes and this targets p27^{KIP} for ubiquitylation and destruction by the proteasome (Bryant et al., 2006) (Figure 11-19).

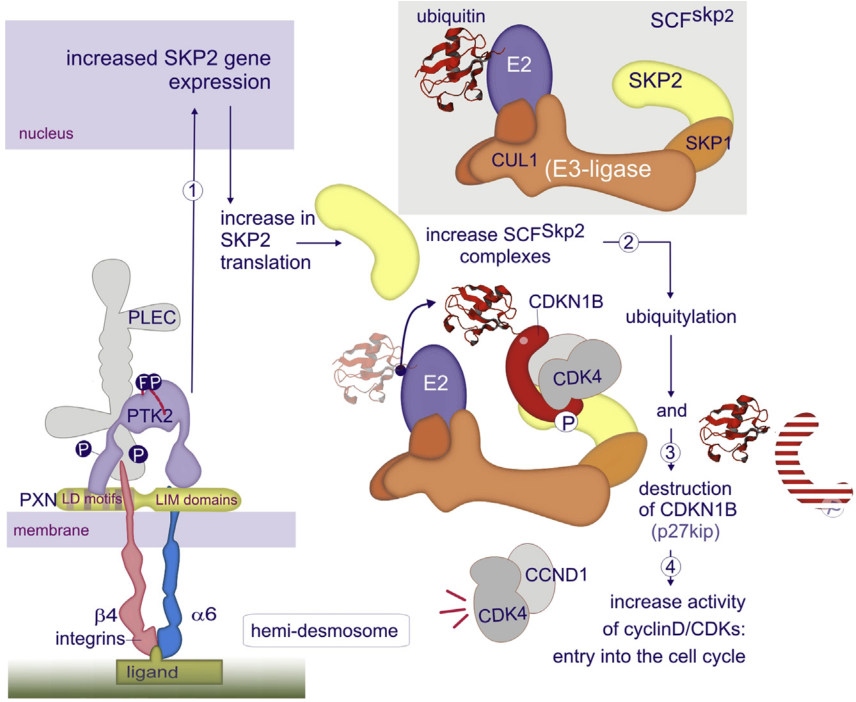

increased SKP2 gene expression
nucleus
①
increase in SKP2 translation
ubiquitin
SCFskp2
E2
SKP2
SKP1
CUL1 (E3-ligase
increase SCFSkp2 complexes
②
ubiquitylation
PLEC
CDKN1B
E2
CDK4
P
and
③
PTK2
P
P
PXN LD motifs
LIM domains
membrane
destruction of CDKN1B (p27kip)
④
CCND1
CDK4
increase activity of cyclinD/CDKs: entry into the cell cycle
β4 integrins
α6
hemi-desmosome
ligand

FIGURE 11-19 Regulation of destruction of p27^{KIP} by the integrin-signaling complex. Increased expression of SKP2, due to activated PTK2K, enhances the formation of the ubiquitin ligase complex SCF^{Skp2}. SKP2 is the receptor component of this complex and it selectively binds (phosphorylated) p27^{KIP} (CDKN1B) leading to its poly-ubiquitylation and destruction. The cells thus lose a cell cycle inhibitor; so favoring entry into G1 phase.

PTK2-mediated activation of growth factor receptors

While growth factor receptors certainly play a role in the activation of integrins the reverse is also true (Miranti and Brugge, 2002; Wang et al., 1996). A good example is the interaction between the integrin αVβ3 and the epidermal growth factor receptor (EGFR/ERBB2) dimer. Adherence to extracellular matrix causes phosphorylation of four tyrosine residues on the ERBB2 in a manner dependent on the adaptor protein BCAR1 and the kinase SRC. The number of receptors activated, and the number of tyrosines phosphorylated is less than that induced by EGF, and there is a failure to achieve a full proliferative response. Addition of EGF then phosphorylates a further tyrosine and so we understand that the full response to low-level stimulation by EGF requires the additional integrin-mediated phosphorylation of the receptor (Giancotti and Tarone, 2003). The importance of this interplay between integrins and growth factor receptors is accentuated by the finding that integrin α6β4 amplifies the transformation of breast cancer cells that overexpress the ERBB2 receptor. This amplification occurs through SRC-mediated phosphorylation of ERBB2 (Figure 11-18) though the tyrosine residue in question is not normally phosphorylated by the neighboring EGFR (Guo et al., 2006).

Cell glycoproteins contribute to integrin signaling. Malignancy is associated with altered expression of glycans and glycoproteins that contribute to the cellular glycocalyx. Indeed, metastatic tumors upregulate expression of bulky glycoproteins. It has been shown that a bulky glycocalyx facilitates integrin clustering by funneling active integrins into adhesion complexes and altering the integrin state by applying tension to matrix-bound integrins. Expression of large tumor-associated glycoproteins in non-transformed mammary cells promoted focal adhesion assembly and facilitated integrin-dependent growth factor signaling to support cell growth and survival. Clinical studies revealed that large glycoproteins are abundantly expressed on circulating tumor cells from patients with advanced disease (Paszek et al., 2014).

SIGNALING FROM CADHERIN CLUSTERS

When cadherins, already bound to β-catenin, insert into the lateral membrane and cluster upon contact with cadherins from neighboring cells, they bring about a series of events that organize the cytoskeleton, both actin and tubulin, and induce a cell-survival signal. The nascent cytoskeleton, in turn, plays an essential role in the maturation of the adherens' junction (Perez et al., 2008; Hong et al., 2013). For instance, the connection with microtubules plays an essential role in the recruitment of myosin II, an event necessary for the subsequent accumulation of E-cadherin and for the formation of contractile actin networks that constitute the zonula adherens (Stehbens et al., 2006). Essential in the process is the activation of phosphatidylinositol 3-kinase and the monomeric GTPase RAC1. Activation of phosphatidylinositol 3-kinase occurs through its recruitment at the membrane through an as yet poorly defined mechanism. Phosphorylation of DLGAP5 (a disk-large homolog bound to cadherin) by the tyrosine-kinase SRC has been reported as one possible mechanism (Laprise et al., 2004). The phosphotyrosine on DLGAP5 is recognized by the SH2 domain of the p85 regulatory subunit (PIK3R1) of phosphatidylinositol 3-kinase (Figure 11-20). The ensuing production of phosphatidylinositol-3,4-bisphosphate or phosphatidylinositol-3,4,5-trisphosphate causes the recruitment of TIAM1, a guanine exchange factor for RAC1 (and CDC42) (Kovacs et al., 2002; Rivard, 2009; Ceccarelli et al., 2007; Habets et al., 1994). Alternatively, RAC1 is activated through VAV2, aRhoGEF that has been reported to associate with δ-catenin (Noren et al., 2000). GTP-bound RAC1 (active) binds the GRD domain of IQGAP, a big scaffold protein that also interacts with APC and CIP170, which are microtubule-associated proteins (Watanabe et al., 2004). After having captured the microtubules at the cell junction, the kinesin motor protein, moving on microtubules toward the plus end, contributes to the provision of proteins, transported in membrane vesicles, and mRNA (necessary for the local production of cytoplasmic proteins).

GTP-bound RAC1 also orchestrates the nucleation of new actin filaments. It binds and activates the regulatory WAVE complex (SRA1/NAP1/ABI/WAVE) of which WAVE modifies the configuration of the ARP2/3 actin-binding protein complex (Ryun et al., 2009; Insall and Davidson, 2011; Chen et al., 2010). This complex of seven proteins contains two actin-related proteins (ARPs) that serve as primers (docking sites) in the process of actin polymerization (process of filament formation). Stabilized actin filaments bind to α-catenin (CTNNA1) and together with myosin II (A/B) they form a contractile network just underneath the plasma membrane (zonula adherens).

The production of 3-inositol phosphorylated phosphatidylinositol also causes the recruitment of the protein kinases PDK1 and AKT1, both through their PH domains, and this causes the activation of AKT1. As shown in Figure 11-17 subsequent phosphorylation of a substantial number of proteins prevents the activation of the apoptotic pathway. This is yet another example of how adhesion molecules provide the cell

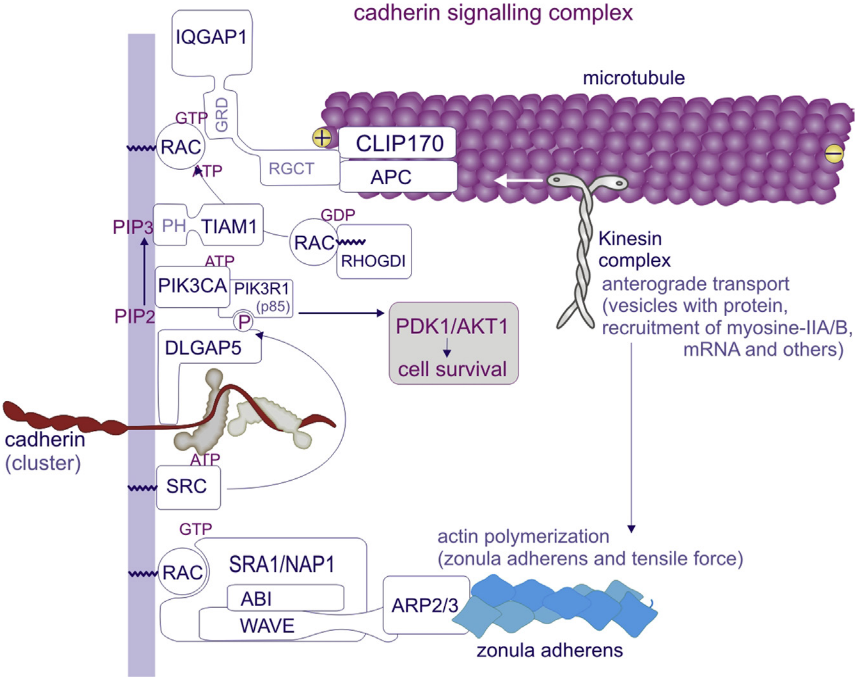


FIGURE 11-20 Cadherin-mediated signaling complexes. Cadherin clusters cause the binding of DLGAP5 to the membrane proximal region. Upon phosphorylation by SRC, DLGAP5 recruits the phosphatidylinositol 3-kinase (PIK3CA) via the SH2 domain of the regulatory subunit (PIK3R1). The ensuing production of PI3,4,5-P3 leads to binding of the PH domain of TIAM1. The nucleotide exchange factor activates RAC1 and this causes the binding of microtubules (IQGAP1 mediated) and the nucleation of actin filaments (ARP2/3 mediated). The microtubules play an essential role in maturation of the junction and in the provision of myosin-II. Actin filament intercalated by myosin-II and held together by α-actinin constitute the zonula adherens.

with important information about their connectedness. Such signals are vital for a coherent functioning of cells within tissues. Return to Chapter 2, “An Introduction to Signal Transduction,” Section “Cells need ways to create symbolic representations of their (changing) environment.” To illustrate how positive signals are always counterbalanced by negative signals, we end this chapter by reporting that E-cadherin also facilitates Death Receptor-induced apoptosis. By forming clusters with death receptor-4 or -5, it facilitates the formation of the death receptor-induced signaling complex (DISC). This may be of great importance in warranting tissue homeostasis (correctly balancing cell proliferation against cell death) (Lu et al., 2014). For a recent review about the many factors that control cell death we refer to Green et al. (2014).

References

Alon, R., Chen, S., Fuhlbrigge, R., Puri, K.D., Springer, T.A., 1998. The kinetics and shear threshold of transient and rolling interactions of L-selectin with its ligand on leukocytes. Proc. Natl. Acad. Sci. U.S.A. 95, 11631–11636.

Angata, T., Tabuchi, Y., Nakamura, K., Nakamura, M., 2007. Siglec-15: an immune system Siglec conserved throughout vertebrate evolution. Glycobiology 17, 838–884.

Aplin, A.E., Stewart, S.A., Assoian, R.K., Juliano, R.L., 2001. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. 153, 273–282.

Aruffo, A., Stamenkovic, I., Milnick, M., Underhill, C.B., Seed, B., 1990. CD44 is the principal cell surface receptor for hyaluronate. Cell 6, 1303–1313.

Assoian, R.K., 1997. Anchorage-dependent cell cycle progression. J. Cell Biol. 136, 1–4.

Bailly, P., Hermand, P., Callebaut, I., Sonneborn, H.H., Khamlichi, S., Mornon, J.P., Cartron, J.P., 1994. The LW blood group glycoprotein is homologous to intercellular adhesion molecules. Proc. Natl. Acad. Sci. U.S.A. 91, 5306–5310.

Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Jones, M., Jackson, D.G., 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J. Cell Biol. 144, 789–901.

Barreiro, O., Yanez-Mo, M., Serrador, J.M., Montoya, M.C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H., Sanchez-Madrid, F., 2002. Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. J. Cell Biol. 157, 1233–1245.

Boller, K., Vestweber, D., Kemler, R., 1985. Cell-adhesion molecule uvomorulin is localized in the intermediate junctions of adult intestinal epithelial cells. J. Cell Biol. 100, 327–332.

Bottazzi, M.E., Zhu, X., Bohmer, R.M., Assoian, R.K., 1999. Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. J. Cell Biol. 146, 1255–1264.

Brackenbury, R., Thiery, J.P., Rutishauser, U., Edelman, G.M., 1977. Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. J. Biol. Chem. 252, 6835–6840.

Brakebusch, C., Fassler, R., 2003. The integrin-actin connection, an eternal love affair. EMBO J. 22, 2324–2333.

Brinkman, A., van der Flier, S., Kok, E.M., Dorssers, L.C., 2000. BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. J. Natl. Cancer Inst. 92, 112–120.

Brown, M.C., Turner, C.E., 2004. Paxillin: adapting to change. Physiol. Rev. 84, 1315–1339.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96, 857–868.

Bryant, P., Zheng, Q., Pumiglia, K., 2006. Focal adhesion kinase controls cellular levels of p27/Kip1 and p21/Cip1 through Skp2-dependent and -independent mechanisms. Mol. Cell Biol. 26, 4201–4213.

Burridge, K., Connell, L.A., 1983. New protein of adhesion plaques and ruffling membranes. J. Cell Biol. 97, 359–367.

Calderwood, D.A., Zent, R., Grant, R., Rees, D.J., Hynes, R.O., Ginsberg, M.H., 1999. The talin head domain binds to integrin-β subunit cytoplasmic tails and regulates integrin activation. J. Biol. Chem. 274, 28071–28704.

Calderwood, D.A., Campbell, I.D., Critchley, D.R., 2013. Talins and kindlins: partners in integrin-mediated adhesion. Nat. Rev. Mol. Cell Biol. 14, 503–517.

Carman, C.V., Springer, T.A., 2003. Integrin avidity regulation: are changes in affinity and conformation underemphasized. Curr. Opin. Cell Biol. 15, 547–556.

Ceccarelli, D.F., Blasutig, I.M., Goudreault, M., Li, Z., Ruston, J., Pawson, T., Sicheri, F., 2007. Non-canonical interaction of phosphoinositides with pleckstrin homology domains of Tiam1 and ArhGAP9. J. Biol. Chem. 282, 13864–13874.

Chen, C.P., Posy, S., Ben-Shaul, A., Shapiro, L., Honig, B.H., 2005. Specificity of cell-cell adhesion by classical cadherins: critical role for low-affinity dimerization through beta-strand swapping. Proc. Natl. Acad. Sci. U.S.A. 102, 8531–8536.

Chen, Z., Borek, D., Padrick, S.B., Gomez, T.S., Metlagel, Z., Ismail, A.M., Umetani, J., Billadeau, D.D., Otwinowski, Z., Rosen, M.K., 2010. Structure and control of the actin regulatory WAVE complex. Nature 468, 533–538.

Clark, E.A., King, W.G., Brugge, J.S., Symons, M., Hynes, R.O., 1998. Integrin-mediated signals regulated by members of the rho family of GTPases. J. Cell Biol. 142, 573–586.

Collins, B.E., Smith, B.A., Bengtson, P., Paulson, J.C., 2006. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. Nat. Immunol. 7, 199–206.

Critchley, D.R., 2009. Biochemical and structural properties of the integrin-associated cytoskeletal protein talin. Annu. Rev. Biophys. 38, 235–254.

Crocker, P., Paulson, J., Varki, A., 2007. Siglecs and their roles in the immune system. Nat. Rev. Immunol. 7, 255–266.

Cummins, P.M., 2012. Occludin: one protein, many forms. Mol. Cell Biol. 32, 242–250.

Curran, T., D'Arcangelo, G., 1998. Role of reelin in the control of brain development. Brain Res. Brain Res. Rev. 26, 285–294.

Davignon, D., Martz, E., Reynolds, T., Kurzinger, K., Springer, T.A., 1981. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. Proc. Natl. Acad. Sci. U.S.A. 78, 4535–4539.

Day, J., 1999. The structure and regulation of hyaluronan-binding proteins. Biochem. Soc. Trans. 27, 115–121.

Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L., Coffer, P.J., 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. 10, 1201–1204.

Dijkers, P.F., Birkenkamp, K.U., Lam, E.W., Thomas, N.S., Lammers, J.W., Koenderman, L., Coffer, P.J., 2002. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J. Cell Biol. 156, 531–542.

Downward, J., 1998. Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. 10, 262–267.

Downward, J., 2004. PI 3-kinase, Akt and cell survival. Semin. Cell Dev. Biol. 15, 177–182.

Ducibella, T., Anderson, E., 1975. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. Dev. Biol. 47, 45–58.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., Springer, T.A., 1986. Induction by IL 1 and interferon-g: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. 137, 245–254.

Edelman, G.M., 1983. Cell adhesion molecules. Science 219, 450–457.

Farquhar, M.G., Palade, G.E., 1963. Junctional complexes in various epithelia. J. Cell Biol. 17, 375–412.

de Fougerolles, A.R., Springer, T.A., 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. 175, 185–190.

Franke, B., van Triest, M., de Bruijn, K.M., van Willigen, G., Nieuwenhuis, H.K., Negrier, C., Akkerman, J.W., Bos, J.L., 2000. Sequential regulation of the small GTPase Rap1 in human platelets. Mol. Cell Biol. 20, 779–785.

Frisch, S.M., Francis, H., 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. 124, 619–626.

Frisch, S.M., Vuori, K., Ruoslahti, E., Chan-Hui, P.Y., 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. 134, 793–799.

Gallin, W.J., Edelman, G.M., Cunningham, B.A., 1983. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. Proc. Natl. Acad. Sci. U.S.A. 80, 1038–1042.

Genot, E., Arrieumerlou, C., Ku, G., Burgering, B.M., Weiss, A., Kramer, I.M., 2000. The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. Mol. Cell Biol. 20, 5469–5478.

Giancotti, F.G., Tarone, G., 2003. Positional control of cell fate through joint integrin/receptor protein kinase signaling. Annu. Rev. Cell Dev. Biol. 19, 173–206.

Gilmore, A.P., Burridge, K., 1996. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. Nature 381, 531–535.

Goldstein, L.A., Zhou, D.F., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F., Butcher, E.C., 1989. human lymphocyte homing receptor, the hermes antigen, is related to cartilage proteoglycan core and link proteins. Cell 56, 1063–1072.

Gowans, J.L., Knight, E.J., 1964. The route of recirculation of lymphocytes in the rat. Proc. R. Soc. Lond. Ser. B 159, 257–282.

Green, D.R., Galluzzi, L., Kroemer, G., 2014. Metabolic control of cell death. Science 345, 1250256 1–11.

Gunthert, U., Hofmann, M., Rudy, Y., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., Herrlich, P., 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65, 13–24.

Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W.J., Inghirami, G., Giancotti, F.G., 2006. Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. Cell 126, 489–502.

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A., Collard, J.G., 1994. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell 77, 537–549.

Hall, J.E., Fu, W., Schaller, M.D., 2011. Focal adhesion kinase: exploring Fak structure to gain insight into function. Int. Rev. Cell Mol. Biol. 288, 185–218.

Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T., Matsuda, M., 2006. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. Mol. Cell Biol. 16, 1770–1776.

Hato, T., Pampori, N., Shattil, S.J., 1998. Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin alphaIIb beta3. J. Cell Biol. 141, 1685–1695.

Hoellerer, M.K., Noble, M.E., Labesse, G., Campbell, I.D., Werner, J.M., Arold, S.T., 2003. Molecular recognition of paxillin LD motifs by the focal adhesion targeting domain. Structure 11, 1207–1217.

Hong, S., Troyanovsky, R.B., Troyanovsky, S.M., 2013. Binding to F-actin guides cadherin cluster assembly, stability, and movement. J. Cell Biol. 201, 131–143.

Huang, M.T., Mason, J.C., Birdsey, G.M., Amsellem, V., Gerwin, N., Haskard, D.O., Ridley, A.J., Randi, A.M., 2005. Endothelial intercellular adhesion molecule (ICAM)-2 regulates angiogenesis. Blood 106, 1636–1643.

Insall, R.H., Davidson, A.J., 2011. Actin-based motility: WAVE regulatory complex structure reopens old SCARs. Curr. Biol. 21, 666–668.

Ishiyama, N., Lee, S.H., Liu, S., Li, G.Y., Smith, M.J., Reichardt, F.L., Ikura, M., 2010. Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion. Cell 141, 117–128.

Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., Tsukita, S., 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. J. Cell Biol. 147, 1351–1363.

Ivetic, A., Ridley, A.J., 2004. The telling tail of L-selectin. Biochem. Soc. Trans. 32, 1118–1121.

Iwahara, T., Akagi, T., Fujitsuka, Y., Hanafusa, H., 2004. CrkII regulates focal adhesion kinase activation by making a complex with Crk-associated substrate, p130Cas. Proc. Natl. Acad. Sci. U.S.A. 101, 17693–17698.

Jones, C., Virji, M., Crocker, P.R., 2003. Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. Mol. Microbiol. 49, 1213–1225.

Katagiri, K., Maeda, A., Shimonaka, M., Kinashi, T., 2003. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. Nat. Immunol. 4, 741–748.

Khwaja, A., Rodriguez, V.P., Wennstrom, S., Warne, P.H., Downward, J., 1997. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J. 16, 2783–2793.

Kilgannon, P., Turner, T., Meyer, J., Wisdom, W., Gallatin, W.M., 1998. Mapping of the ICAM-5 (telencephalin) gene, a neuronal member of the ICAM family, to a location between ICAM-1 and ICAM-3 on human chromosome 19p13.2. Genomics 54, 328–330.

King, W.G., Mattaliano, M.D., Chan, T.O., Tsichlis, P.N., Brugge, J.S., 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol. Cell Biol. 17, 4406–4418.

Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., Matsuda, M., 1998. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev. 12, 3331–3336.

Kovacs, E.M., Ali, R.G., McCormack, A.J., Yap, A.S., 2002. E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. J. Biol. Chem. 277, 6708–6718.

Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B., Boussiotis, V.A., 2004. RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell 7, 585–595.

Laprise, P., Viel, A., Rivard, N., 2004. Human homolog of disc-large is required for adherens junction assembly and differentiation of human intestinal epithelial cells. J. Biol. Chem. 279, 10157–10166.

Lasky, L.A., Singer, M.S., Yednock, T.A., Dowbenko, D., Fennie, C., Rodriguez, T., Stachel, S., Rosan, S.D., 1989. Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell 56, 1045–1055.

Laurent, T.C., Fraser, J.R., 1992. Hyaluronan. FASEB J. 6, 2397–2404.

Law, S.K., Tan, S.M., Ranganathan, S., Cheng, M., 2004. The integrin alpha L beta 2 hybrid domain serves as a link for the propagation of activation signal from its stalk regions to the I-like domain. J. Biol. Chem. 279, 54334–54339.

Lawson, C., Lim, S.T., Uryu, S., Chen, X.L., Calderwood, D.A., Schlaepfer, D.D., 2012. FAK promotes recruitment of talin to nascent adhesions to control cell motility. J. Cell Biol. 196, 223–232.

Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., Pouyssegur, J., 1993. Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. J. Cell Biol. 122, 1079–1088.

Li, H., Leung, T.C., Hoffman, S., Balsamo, J., Lilien, J., 2000. Coordinate regulation of cadherin and integrin function by the chondroitin sulfate proteoglycan neurocan. J. Cell Biol. 149, 1275–1288.

Lilien, J., Balsamo, J., 2005. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. Curr. Opin. Cell Biol. 17, 459–465.

Lim, Y., Han, I., Jeon, J., Park, H., Bahk, Y.Y., Oh, E.S., 2004. Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. J. Biol. Chem. 279, 29060–29065.

Lu, M., Marsters, S., Ye, X., Luis, E., Gonzalez, L., Ashkenazi, 2014. E-cadherin couples death receptors to the cytoskeleton to regulate apoptosis. Mol. Cell 54, 987–998.

Mailleux, A.A., Overholtzer, M., Schmeizie, T., Bouillet, P., Strasser, A., Brugge, J.S., 2007. BIM regulates apoptosis during mammary ductal morphogenesis and its absence reveals alternative cell death mechanisms. Dev. Cell 12, 221–234.

Majewski, N., Nogueira, V., Bhaskar, P., Coy, P.E., Skeen, J.E., Gottlob, K., Chandel, N.S., Thompson, C.B., Robey, R.B., Hay, N., 2004. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol. Cell 16, 819–830.

Marshall, B.T., Long, M., Piper, J.W., Yago, T., McEver, R.P., Zhu, C., 2003. Direct observation of catch bonds involving cell-adhesion molecules. Nature 423, 190–193.

Miranti, C.K., Brugge, J.S., 2002. Sensing the environment: a historical perspective on integrin signal transduction. Nat. Cell Biol. 4, E83–E90.

Mitra, S.K., Mikolon, D., Molina, J.E., Hsia, D.A., Hanson, D.A., Chi, A., Lim, S.T., Bernard-Trifilo, J.A., Ilic, D., Stupack, D.G., Cheresh, D.A., Schlaepfer, D.D., 2006. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. Oncogene 25, 5969–5984.

Miyamoto, S., Teramoto, H., Coso, O.A., Gutkind, J.S., Burbelo, P.D., Akiyama, S.K., Yamada, K.M., 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131, 791–805.

Modur, V., Nagarajan, R., Evers, B.M., Milbrandt, J., 2002. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J. Biol. Chem. 277, 47928–47937.

Mould, A.P., Humphries, J.H., 2004. Adhesion articulated. Nature 432, 27–28.

Niessen, C.M., Leckband, D., Yap, A.S., 2011. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. Physiol. Rev. 91, 691–731.

Nishizumi, H., Horikawa, K., Mlinaric-Rascan, I., Yamamoto, T., 1998. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. J. Exp. Med. 187, 1343–1348.

Nitschke, L., 2005. The role of CD22 and other inhibitory co-receptors in B-cell activation. Curr. Opin. Immunol. 17, 290–297.

Noren, N.K., Liu, B.P., Burridge, K., Kreft, B., 2000. P120 Catenin regulates the actin cytoskeleton via Rho family GTPase. J. Cell Biol. 150, 567–580.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J., Matsuda, M., 2001. Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. EMBO J. 20, 333–3341.

O'Keefe, T.L., Williams, G.T., Davies, S.L., Neuberger, M.S., 1996. Hyperresponsive B cells in CD22-deficient mice. Science 274, 798–801.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi, R.G., Lobb, R., 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59, 1203–1211.

Ozawa, M., Baribault, H., Kemler, R., 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. 8, 1711–1717.

Paszek, M.J., DuFort, C.C., Rossier, O., Bainer, B., Mouw, J.K., Godula, K., Hudak, J.E., Lakins, J.N., Wijekoon, A.C., Cassereau, L., Rubashkin, M.G., et al., 2014. The cancer glycocalyx mechanically primes integrin-mediated growth and survival. Nature 511, 319–325.

Patel, S.D., Ciatto, C., Chen, C.P., Bahna, F., Rajebhosale, M., Arkus, N., Schieren, I., Jessell, T.M., Honig, B., Price, S.R., Shapiro, L., 2006. Type II cadherin ectodomain structures: implications for classical cadherin specificity. Cell 124, 1255–1268.

Perez, T.D., Tamada, M., Sheetz, M.P., Nelson, W.J., 2008. Immediate-early signaling induced by E-cadherin engagement and adhesion. J. Biol. Chem. 283, 5014–5022.

Plas, D.R., Rathmell, J.C., Thompson, C.B., 2002. Homeostatic control of lymphocyte survival: potential origins and implications. Nat. Immunol. 3, 515–521.

Powell, L.D., Varki, A., 1995. I-type lectins. J. Biol. Chem. 2720, 14243–14246.

Prutzman, K.C., Gao, G., King, M.L., Iyer, V.V., Mueller, G.A., Schaller, M.D., Campbell, S.L., 2004. The focal adhesion targeting domain of focal adhesion kinase contains a hinge region that modulates tyrosine 926 phosphorylation. Structure 12, 881–891.

Raab, M., Wang, H., Lu, Y., Smith, X., Wu, Z., Strebhardt, K., Ladbury, J.E., Rudd, C.E., 2010. T cell receptor "inside-out" pathway via signaling module SKAP1-RapL regulates T cell motility and interactions in lymph nodes. Immunity 32, 541–556.

Reedquist, K.A., Ross, E., Koop, E.A., Wolthuis, R.M., Zwartkruis, F.J., van Kooyk, Y., Salmon, M., Buckley, C.D., Bos, J.L., 2000. The small GTPase, Rap1, mediates CD31-induced integrin adhesion. J. Cell Biol. 148, 1151–1158.

Reginato, M.J., Mills, K.R., Paulus, J.K., Lynch, D.K., Sgrol, D.C., Debnath, J., Muthswamy, S.K., 2003. Integrins and EGFR co-ordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat. Cell Biol. 5, 733–740.

Rhee, J., Mahfooz, N.S., Arregui, C., Lilien, J., Balsamo, J., VanBerkum, M.F., 2002. Activation of the repulsive receptor Roundabout inhibits N-cadherin-mediated cell adhesion. Nat. Cell Biol. 4, 798–805.

Rivard, N., 2009. Phosphatidylinositol 3-kianse: a key regulator in adherens junction formation and function. Front. Biosci. 14, 510–522.

Rosen, S.D., 2004. Ligands for L-selectin: homing, inflammation, and beyond. Annu. Rev. Immunol. 22, 129–156.

Ruoslahti, E., Pierschbacher, M.D., 1987. New perspectives in cell adhesion: RGD and integrins. Science 238, 491–497.

Ryun, J.R., Echarri, A., Li, R., Pendergast, A.M., 2009. Regulation of cell-cell adhesion by Abi/Diphanous complexes. Mol. Cell Biol. 29, 1735–1748.

Saito, M., Tucker, D.K., Kohlhorst, D., Niessen, C.M., Kowalczyk, A.P., 2012. Classical and desmosomal cadherins at a glance. J. Cell Sci. 125, 2547–2552.

Sanchez-Pulido, L., Martin-Belmonte, F., Valencia, A., Alonso, M.A., 2002. MARVEL: conserved domain involved in membrane apposition events. Trends Biochem. Sci. 27, 599–601.

Shen, Y., Jia, Z., Nagele, R.G., Ichikawa, H., Goldberg, G.S., 2006. SRC uses Cas to suppress Fhl1 in order to promote non-anchored growth and migration of tumor cells. Cancer Res. 66, 1543–1552.

Shimaoka, M., Takagi, J., Springer, T.A., 2002. Conformational regulation of integrin structure and function. Annu. Rev. Biophys. Biomol. Struct. 31, 485–516.

Smith, D.K., Xue, H., 1997. Sequence profiles of immunoglobulin and immunoglobulin-like domains. J. Mol. Biol. 274, 530–545.

Springer, T.A., Galfre, G., Secher, D.S., Milstein, C., 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. 9, 301–306.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76, 301–314.

Stehbens, S.J., Paterson, A.D., Crampton, M.S., Shewan, A.M., Ferguson, C., Akhmanova, A., Parton, R.G., Yap, A.S., 2006. Dynamic microtubules regulate the local concentration of E-cadherin at cell-cell contacts. J. Cell Sci. 119, 1801–1811.

Stoolman, L.M., Rosen, S.D., 1983. Possible role for cell-surface carbohydrate-binding molecules in lymphocyte recirculation. J. Cell Biol. 96, 722–729.

Suenaga, T., Satoh, T., Somboonthum, P., Kawaguchi, Y., Arase, H., 2010. Myelin-associated glycoprotein mediates membrane fusion and entry of neurotropic herpesviruses. Proc. Natl. Acad. Sci. U.S.A. 107, 866–871.

Tadokoro, S., Shattil, S.J., Eto, K., Tai, V., Liddington, R.C., de Pereda, J.M., Ginsberg, M.H., Calderwood, D.A., 2003. Talin binding to integrin-β tails: a final common step in integrin activation. Science 302, 103–106.

Tamkun, J.W., DeSimone, D.W., Fonda, D., Patel, R.S., Buck, C., Horwitz, A.F., Hynes, R.O., 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 46, 271–282.

Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matuoka, K., Takenawa, T., Kurata, T., Nagashima, K., 1994. C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. Proc. Natl. Acad. Sci. U.S.A. 91, 3443–3447.

Tanentzapf, G., Brown, N.H., 2006. An interaction between integrin and the talin FERM domain mediates integrin activation but not linkage to the cytoskeleton. Nat. Cell Biol. 8, 601–606.

Tsukita, S., Oishi, K., Sato, N.J., Sagara, J., Kawai, A., 1994. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. 126, 391–401.

Turksen, K., Troy, T.C., 2004. Barriers built on claudins. J. Cell Sci. 117, 2435–2447.

Van Itallie, C.M., Anderson, J.M., 2006. Claudins and epithelial paracellular transport. Annu. Rev. Physiol. 68, 403–429.

Van Itallie, C.M., Anderson, J.M., 2014. Architecture of tight junctions and principles of molecular composition. Sem. Cell Dev. Biol. 36, 157–165.

Vavylonis, D., Kovar, D.R., O'Shaughnessy, B., Pollard, T.D., 2006. Model of formin-associated actin filament elongation. Mol. Cell 21, 455–466.

Wang, R., Kobayashi, R., Bishop, J.M., 1996. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. Proc. Natl. Acad. Sci. U.S.A. 93, 8425–8430.

Wary, K.K., Mariotti, A., Zurzolo, C., Giancotti, F.G., 1998. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 94, 625–634.

Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., Kaibuchi, K., 2004. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Dev. Cell 7, 871–883.

Wojciak-Stothard, B., Ridley, A.J., 2003. Shear stress-induced endothelial cell polarization is mediated by Rho and Rac but not Cdc42 or PI 3-kinases. J. Cell Biol. 161, 423–439.

Xiao, T., Takagi, J., Coller, B.S., Wang, J.H., Springer, T.A., 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432, 59–67.

Xiong, J.P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D.L., Joachimiak, A., Goodman, S.L., Arnaout, M.A., 2001. Crystal structure of the extracellular segment of integrin alpha Vbeta3. Science 294, 339–345.

Yago, T., Wu, J., Wey, C.D., Klopocki, A.G., Zhu, C., McEver, R.P., 2004. Catch bonds govern adhesion through L-selectin at threshold shear. J. Cell Biol. 166, 913–923.

Ye, F., Hu, G., Taylor, D., Ratnikov, B., Bobkov, A.A., McLean, M.A., Sligar, S.G., Taylor, K.A., Ginsberg, M.H., 2010. Recreation of the terminal events in physiological integrin activation. J. Cell Biol. 188, 157–173.

Yoshida, C., Takeichi, M., 1982. Teratocarcinoma cell adhesion: identification of a cell-surface protein involved in calcium-dependent cell aggregation. Cell 28, 217–224.

Zaidel-Bar, R., Itzkovitz, S., Ma'ayan, A., Iyengar, R., Geiger, B., 2007. Functional atlas of the integrin adhesome. Nat. Cell Biol. 9, 858–867.

Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., Hung, M.C., 2001. HER-2/neu induces p53 ubiquitinylation via Akt-mediated MDM2 phosphorylation. Nat. Cell Biol. 3, 973–982.

CHAPTER

12

WNT Signaling and the Regulation of Cell Adhesion and Differentiation

Here we consider how an essential component of adherent junctions, β-catenin (CTNNB1) or plakoglobin (JUP), can switch from a structural component to a transcription factor capable of changing the cell transcriptome and as a consequence its phenotype. The switch from adhesion to transcription occurs through a signal transduction pathway controlled by the cytokine (or morphogen) WNT. When speaking of WNT we refer to a diverse collection of signaling pathways that trigger and steer cells, depending on the context, in multiple directions: polarity, mobility, proliferation, differentiation, survival, or self-renewal (Figure 12-1). Diversity in Wnt signaling derives from the diversity of its components, a set of 19 ligands, 10 receptors, and multiple co-receptors (van Amerongen and Nusse, 2009). Disorders in the canonical Wnt pathway are revealed in cancer and bone pathologies (Baron and Kneissel, 2013). We will discuss principally the signaling pathway that converges on β-catenin, also known as the canonical Wnt pathway. We elaborate the role of Wnt proteins in epithelial–mesenchymal transition, in the maintenance of the self renewal capacity of stem cells in the crypts of the intestinal tract and in planar cell polarity (PCP).

DESTABILIZATION OF ADHERENS JUNCTIONS CAUSES CELLULAR DEDIFFERENTIATION

The surfaces that separate interior from exterior, such as the skin, the linings of the body cavities, and the gut are formed from tight sheets of epithelial cells. Similarly, the linings of the vasculature, including the heart, blood vessels, and lymphatics, are formed from endothelial cells, which are specialized epithelial cells. Unlike bone, cartilage, or interstitium,

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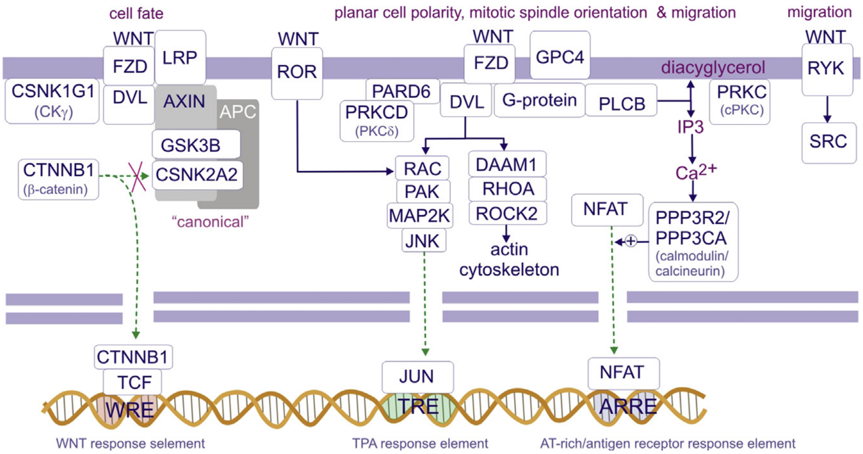


FIGURE 12-1 Schematic representation of Wnt pathways. Schematic representation of the three pathways attributed to WNT. The canonical pathway converges on β-catenin and involves the Wnt-response element. This pathway plays a role in epithelial–mesenchymal transition and lies at the heart of bone pathologies and cancer. The pathway involving RAC, RHOA, and PLCB governs cell planar polarity, migration, and mitotic spindle orientation. Finally, WNT interacts with two members of the tyrosine protein kinase receptors (ROR and RYK), but the downstream events are still poorly characterized.

the extracellular matrix is minimal, comprising a thin sheet, the basal lamina. It is the cells themselves that bear the brunt of physical stresses such as the peristalsis of the gut and the pulsatile movement of the vessels. Tissue structure is maintained by an elaborate series of cellular attachments, tight junctions (also known as zonula occludens), adherens junctions (forming the zonula adherens), and desmosomes (Latin *occludo* to block up; *desmos* meaning band, ligament) (return to Chapter 11, "Signal Transduction to and from Adhesion Molecules," Figure 11-11).

If the adherens junctions stabilizing a cell layer are artificially destabilized, for instance by antibodies against cadherin, there is a loss of cell polarity and the particular spatial distribution of the membrane proteins becomes randomized. Insulin-like growth factor receptor type-I (IGF1R, normally disposed on the basal surface) and the glucose transporters (SLC5A1, on the apical surface) are now distributed all over the cell surface. The tight junctions disassemble through detachment of the zonula adherens-associated proteins (TJP1) and the link between the E-cadherins (CDH1) and the catenins is broken; TJP proteins are scattered into the cytosol (Fialka et al., 1996) and, importantly, β-catenin (CTNNB1) is present in the nucleus (Eger et al., 2000) (Figure 12-1). The cells produce more fibronectin (extracellular matrix), and switch from the production of keratin to vimentin (both forming intermediate filaments). In short they begin to resemble fibroblasts

TABLE 12-1 Markers of epithelial–mesenchymal transition

Changes in epithelial cells during epithelial–mesenchymal transition (EMT)	
Gain of expression	Fibronectin (FN1), vimentin (VMT), SNAI1, SNAI2 (slug), TWIST1, goosecoid (GSC), FOXC2, SOX10, MMP9, MMP3, integrin$\alpha V\beta 6$ (ITGAV, ITGB6), ZEB1, ZEB2, N-cadherin (CDH2), PDGFR, PRKCA, PLCG, FOSL
Loss of expression	E-cadherin (CDH1), desmoplakin (DSP), occluding (OCLN), keratin (KRT, intermediate filament)
Increase in activity	Integrin-linked kinase (ILK), GSK3B, SNAI2, TWIST1, ZEB1, ZEB2
Accumulation in the nucleus	β-catenin (CTNNB1), SMAD2/3, SNAI1, SNAI2, ZEB1, ZEB2
Functional markers	Increased migration, increased matrix invasion, increased scattering, elongation of cell shape, resistance to anoïkis

From Ref. Lee et al. (2006).

(mesenchymal cells). For changes in gene expression, protein activity, and protein localization see Table 12-1 (subject also discussed in Chapter 9, “Protein Kinase C in Oncogenic Cell Transformation and Cell Polarity,” Section “Protein kinase C contributes to cell transformation by driving the epithelial–mesenchymal transition”).

This disorientation also referred to as the epithelial–mesenchymal transition (EMT), plays an important role in the reorganization of tissue in the developing embryo (Hay, 2005; Nakaya and Sheng, 2013). It allows tight sheets of cells to loosen up, to detach and then migrate, and to establish new tissues elsewhere (organ development). Typical examples are gastrulation and neural crest-cell migration. A similar, but uncontrolled transition occurs in the formation of cancer cells as a consequence of transformation of epithelial cells (Lee et al., 2006; Huber et al., 2005) and down-regulation of E-cadherin expression (Berx and van Roy, 2001). The importance of cell–cell contact in the maintenance of the differentiated phenotype was well demonstrated in experiments in which ectopic expression of E-cadherin suppressed the transformation of a colon carcinoma cell line (that normally contains very little E-cadherin) (Figure 12-2) (Gottardi et al., 2001). Loss of E-cadherin can also be brought about by WNT and a similar set of events as described above follows that eventually leads to cell dedifferentiation (Nelson and Nusse, 2004). In certain situations, tissue cells must remain in a dedifferentiated state in order to allow for tissue renewal. Here we are referring to the maintenance of an epithelial stem cell niche in which WNT plays an essential role (Reya and Clevers, 2005).

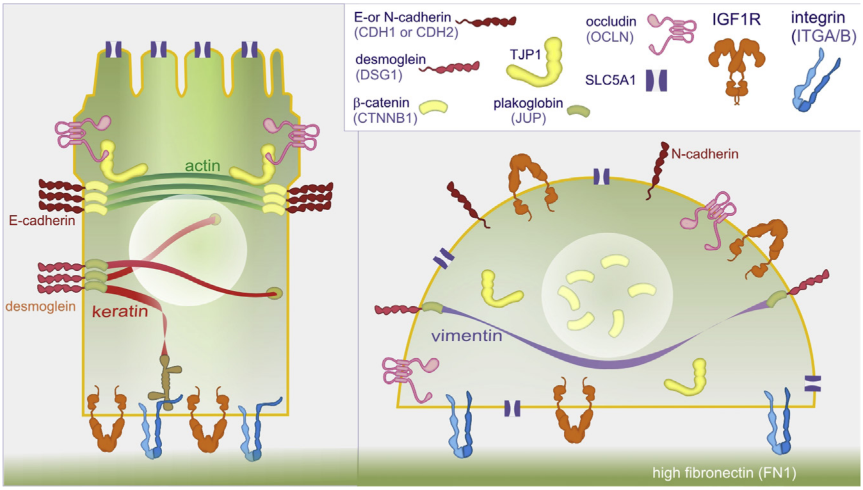


FIGURE 12-2 Loss of cell–cell contact and the dissipation of cell polarity. The polarized state is well illustrated by the selective distribution of the components of the tight- and adherent junctions (lateral surface), with insulin-like growth factor receptor (IGF1R) at the basal surface and the Na^+/glucose symporter (SLC5A1) at the apical surface. With the dissipation of adherent junctions this functional polarization is lost, the Na^+/glucose symporters and IGF receptors being randomly distributed and keratin replaced by vimentin. The cells become dedifferentiated. WNT plays a crucial role in all this.

THE DISCOVERY OF THE WNT FAMILY OF CYTOKINES

The discovery of Wnt arose from several independent lines of research. Work with *Drosophila* brought to light a gene linked to wing development, and given the name *wingless* (Wg) (Sharma and Chopra, 1976). Outright deletion of this gene is lethal, causing defects that are manifest in early development. Wingless and its downstream signaling components are often referred to as “segment polarity genes” (Nusslein-Volhard and Wieschaus, 1980). We now know that the Wnt pathway is used in different stages of *Drosophila* development: in the formation of the segments of the head, thorax, and trunk; in the development of appendages such as legs and antennae; and in certain aspects of oogenesis and neurogenesis (Gerhart, 1999). It is also operational in the organization of PCP, assisting in the positioning of wing hairs and sensory bristles.

In a totally unrelated investigation it was found that mouse mammary tumors caused by the retrovirus MMTV result from the insertion of the viral DNA within the “int-1 locus” of the mouse genome (viral integration site) (Nusse and Varmus, 1982). Later, it transpired that the int-1 locus and the freshly discovered segment polarity gene Wg bear similar sequences and in fact are homologous genes (Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987). As a result, a new nomenclature, Wnt, was proposed, being an amalgamation of Wg and int-1 (gene name WNT in the human genome) (Nusse et al., 1991). On top of all this, a previously known mouse mutant, *swaying*, also turned out to be an allele of the Wnt-1 gene (Thomas et al., 1991). Due to a malformed cerebellum, these animals manifest a lack of muscular coordination (ataxia), the *swaying* of the head being one of the symptoms. Here, the mutated Wnt-1 protein lacks the carboxy-terminal half due to a single base pair mutation, which creates a premature stop codon.

WNT ALLELES AND DISEASE

WNT may be regarded as a proto-oncogene (having the potential to cause cancer), but so far no human tumors have been correlated with a WNT defect. However, certain components of the WNT-signaling transduction pathway do qualify as true "oncogenes." Dysfunctioning WNT alleles have been found in other human pathologies such as osteoporosis, ostegenesis imperfecta type XV, obesity, type 2 (late onset) diabetes, Mullerian-duct regression, and the embryo-lethal phenotype Tetra-Amelia (lack of extremities) (Moon et al., 2004).

From mutation analysis in *Drosophila* and from screening for homologous genes in mice, it emerged that Wnt forms a large family of genes (at least 19 members in mammals) having different functions and different signal transduction pathways. Human WNT genes are categorized in the "wingless-type MTTV integration site family" in the HUGO gene name classification. As with the RAS–MAP-kinase pathway (return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Section "The RAS signaling pathway"), mutation analysis in genetically accessible organisms greatly facilitated the study of components associated with WNT and operational in the regulation of proliferation, differentiation, and cell fate in mammalian cells.

Following its discovery, Wnt remained a "genetic entity," evading proper definition as a protein for about 15 years. Its effects were studied through gene amplification or deletion, through injection of mRNA or latterly through translation arrest by applying siRNA. Proteins of the Wnt family, 40 kDa in size, are now recognized as secreted palmitoylated glycoproteins (Willert et al., 2003). The lipid attachment, essential for secretion (Franch-Marro et al., 2008) and signaling, renders Wnt amphipathic, meaning that Wnt operates at the interface of a lipid and aqueous environment. Thus, Wnt either remains associated with membranes (Korkut et al., 2009) or with proteins that offer a hydrophobic pocket (such as the

Frizzled receptor to which we return later) (Janda et al., 2012). However, Wnt can diffuse into the tissues, over a distance of up to 30 cells, in this way forming a morphogenic gradient (albeit short ranged) (Panakova et al., 2005). In the context of stem cell niches, Wnt operates in a juxtacrine fashion (acting on neighboring cells) (Sato et al., 2011).

Wnt IN DEVELOPMENT

For an informative review of Wnt effects in *drosophila* see Klingensmith and Nusse (Klingensmith and Nusse, 1994). For a well-illustrated description of Wnt's role in morphogenesis of *Drosophila* see Martinez-Arias (Martinez-Arias, 2003). For developmental disorders in mice Wnt knockouts see Logan and Nusse (Logan and Nusse, 2004).

Read how the discoverers of the int locus view the development of the Wnt field (Nusse and Varmus, 2012).

Web resource

The Wnt homepage provides a continuing update of Wnt and its signal transduction pathways (in human, mouse, fly, and fish), including figures and gene tables. This very informative Web site is maintained by the laboratory of Roel Nusse at Stanford University. URL: http://www.stanford.edu/group/nusselab/cgi-bin/wnt.

WNT SIGNALS THROUGH β-CATENIN

Mutational analysis in *Drosophila* led to the discovery of a signal transduction pathway consisting of the ligand (wingless, Wg), its receptor (frizzled, Fz), and downstream components. By scoring for rescue or aggravation of the phenotype (so-called epistatic tests), these were found to act in the sequence:

disheveled (Dsh) → shaggy (Sgg) → armadillo (Arm) → pangolin (Pan)

The sequence results in the expression of the transcription factor engrailed (en) (Figure 12-3) (Nusslein-Volhard and Wieschaus, 1980; Siegfried et al., 1992; Noordermeer et al., 1994) (for review see Willert and Nusse, 1998). The genes involved are named after the mutant phenotypes they give rise to, having either abnormal hair patterns on their body or wings (as in frizzled, shaggy, or disheveled) or with abnormal larval cuticle formation (armadillo or pangolin) (Figure 12-3).

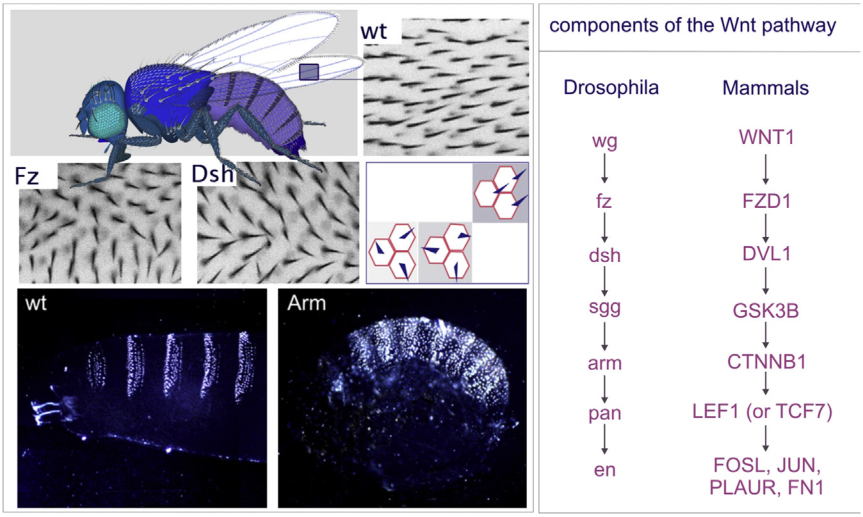


FIGURE 12-3 Examples of *Drosophila* phenotypes due to mutations in the Wnt pathway. In the wild-type fly, actin, which forms a single hair, polymerizes in a defined position with uniform orientation within the plane of the epithelial wing cells. Mutations in Fz and Dsh cause a loss of planar polarity of these cells. Polymerization occurs at random points within the plane of the epithelium giving rise to a frizzled (curly) or disheveled (disorganized) phenotype. Mutations in Arm give rise to a highly segmented embryo resembling the banded shell of an armadillo. *Image of hairs adopted from Wong and Adler, (1993), originally published in the Journal of Cell Biology 123, 209–221. Images of* Drosophila *Wt and Arm are kindly provided by Dr Martinez-Arias, Cambridge, UK.*

What these genes represent and how they are interrelated and integrated into the transduction pathways that determine cell fate or polarity required a number of different experimental setups. Sequence analysis and immunocytochemical experiments provided evidence that armadillo is a homolog of the mammalian β-catenin, a component of the adherens junction, and that it also resembles plakoglobin, a component of desmosomes (Peifer and Wieschaus, 1990; Peifer et al., 1992). Pangolin encodes a protein homologous to the transcription factor, LEF1 (Brunner et al., 1997) that interacts with β-catenin. This interaction is essential for transmission of the Wnt signal into the nucleus (Behrens et al., 1996; Riese et al., 1997). Armadillo also enters the cell nucleus to form a binary transcription complex with pangolin (van de Wetering et al., 1997) (for review, see Clevers and van de Wetering, 1997). The mammalian counterparts of pangolin were initially discovered as nuclear proteins involved in lymphocyte development and in the control of expression of T-cell receptor complex components. They were named lymphoid-enhancer transcription factor-1 (LEF-1) and T-cell factor-1 (TCF1) (van de Wetering et al., 1991; Travis et al., 1991; Waterman et al., 1991). They belong to the TCF family of which humans express four members (see below, “more about TCF family”).

SWITCHING TCF FROM A REPRESSOR TO AN ACTIVATOR

With the list of components in hand it became possible to construct a sequence of events in mammalian cells that lead to the Wnt-mediated changes in gene transcription. In its absence, members of the TCF family associate with Groucho (gene name TLE1) and together they repress DNA transcription. Groucho was named after Julius Henry "Groucho" Marx because its loss-of-function gives rise to flies with bushy eyebrows, resembling the stage appearance of the American comedian (Figure 12-4).

(TLE1 Groucho) causes deacetylation of histones but it is also able to bind histones thereby giving rise to a dense packing of the nucleosomes (known as chromatin condensation). Both events make the gene enhancer and promotor regions concerned inaccessible for regulatory transcription factors and for the RNA-polymerase pre-initiation complex (Cavallo et al., 1998). Return to Chapter 7, "Bringing the Signal into the Nucleus," Section "Transcription and transcription factors," for more information about the regulation of transcription. Entry of β-catenin into the nucleus and subsequent binding to members of the TCF leads to the loss of Groucho and its repressor partners. Instead, the TCF/β-catenin complex acts as a platform for the recruitment of several co-activators. These bring the DNA in a more accessible state and set in train the transcription of numerous genes important for proliferation, differentiation, or the determination of cell fate (depending on cell type and context) (Figure 12-5). In *Drosophila*, Armadillo competes directly with Groucho for the binding to Pangolin, in humans the entry of β-catenin causes a swap in TCF proteins. The TCF7L1/TLE1 (repressor) complex is replaced by the TCF7/β-catenin or LEF1/β-catenin (activator) complex.

FIGURE 12-4 Bushy eyebrows. On the left the comedian Groucho Marx, on the right the Drosophila mutant named after him because of an increased number of supraorbital bristles. Drosophila *image courtesy of Dr Barbara Jennings, University College London, UK.*

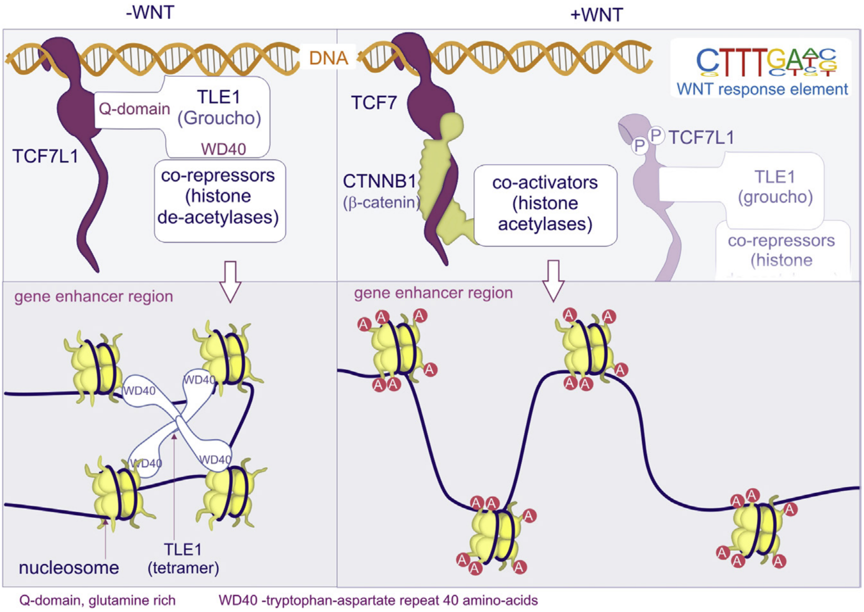


FIGURE 12-5 β-catenin switches the role of TCF from a transcriptional repressor to an activator. TCF7L1 binds to the Q-domain of Groucho (TLE1). This in turn binds, with its WD40 domain, multiple co-repressors among which histone deacetylases. Together they render the enhancer and promoter region of genes poorly accessible for regulatory transcription factors and for the pre-initiation transcription complex. When β-catenin (CTNNB1) enters the nucleus in mammalian cells, it causes a swap of TCF proteins. TCF7L1 is replaced by TCF7 bound to β-catenin. This not only blocks the access of Groucho but also leads to the recruitment of co-activators that render the DNA accessible through relaxation of the nucleosomes. Members of the TCF family bind to the WNT-response element, recognizing the CTTTGA sequence (see inset).

From all this it was understood that β-catenin is localized either at the cell membrane, as part of an adhesion complex with cadherins, or in the nucleus, and that its presence in the nucleus profoundly alters the course of gene expression. It remains to be seen how WNT affects the translocation of β-catenin from the membrane to the nucleus.

ADENOMATOUS POLYPOSIS COLI AND THE REGULATION OF SUBCELLULAR LOCALIZATION OF β-CATENIN

Hints indicating the mechanism by which β-catenin is localized were provided by an inherited disorder, adenomatous polyposis coli (APC), characterized by early onset of numerous polyps in the colon, which progress to malignancy (Lynch and de la Chapelle, 2003). The polyps are a consequence of germ line and (subsequent) somatic mutations that cause truncation of APC, a protein that was named after the disease (Groden et al., 1991) (for review, see Polakis, 2000). Intact APC forms a complex of proteins that interact with β-catenin and determine its destruction, while stimulation of the WNT pathway protects the β-catenin allowing it to accumulate in the cell (Figure 12-6) (Hinck et al., 1994). In *Drosophila*, genetic ablation of Apc causes up-regulation of β-catenin signaling (Ahmed et al., 1998). In humans, a lack of APC and the ensuing nuclear translocation of β-catenin are linked to cell transformation giving rise to colorectal polyps. When APC is overexpressed there is a reduction in the cellular content of β-catenin due to enhanced degradation (Munemitsu et al., 1995).

Note that APC is not to be confused with APC/C (anaphase-promoting complex/cyclosome) an E3-ubiquitin ligase that plays a role in the progression of the cell cycle during mitosis.

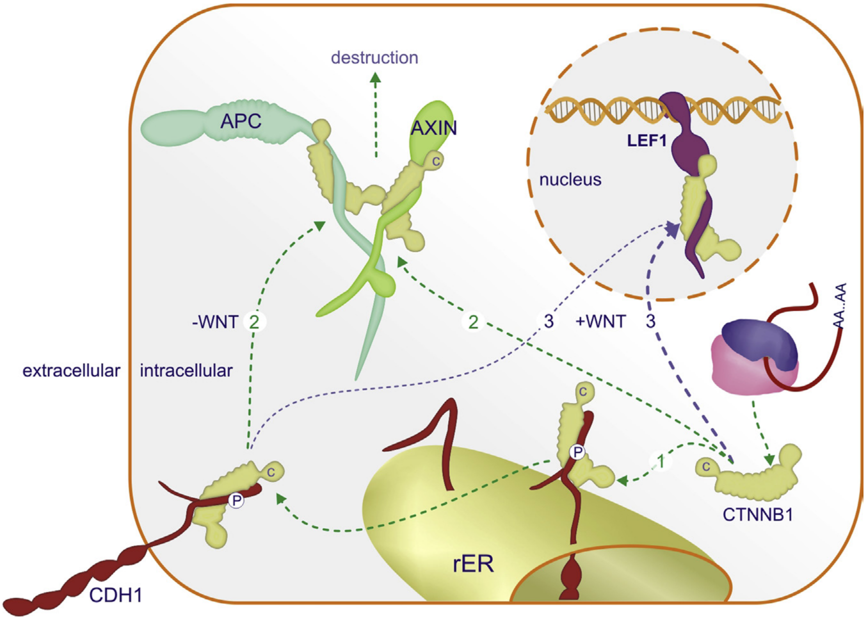


FIGURE 12-6 Which way β-catenin? Following synthesis on free ribosomes, β-catenin binds to the cytosolic segment of cadherin at the level of the rough endoplasmic reticulum (rER) (1) This binding is rendered effective through prior phosphorylation of cadherin by CSNK2A1 and GSK3B. The complex transfers to the plasma membrane where it forms a component of the adherent junctions. Superfluous β-catenin (major source de novo synthesis, minor source released from junctions) is recognized by AXIN and APC (2) This leads to its phosphorylation, ubiquitylation, and subsequent destruction. The presence of WNT disables the destruction complex and β-catenin can now escape into the nucleus, there to bind transcription factors of the TCF/LEF family (3) A change in gene transcription ensues.

The importance of APC has also been established in a transgenic mice model with inducible ablation of the APC gene. Here, Wnt signaling is acutely activated in intestinal epithelial cells. There is copious nuclear accumulation of β-catenin, and this coincides with a transformation toward a "crypt progenitor-like" phenotype (Sansom et al., 2004). Finally, overexpression of β-catenin, through gene transfection, gives rise to a transformed phenotype as found in colorectal cancer (Orford et al., 1999). Thus, Wnt signaling causes an elevated level of β-catenin by disabling the destruction-promoting activity of APC. It is the accumulation of cytosolic-free β-catenin that favors its translocation into the nucleus.

Importantly, intact APC also forms a complex with glycogen synthase kinase-3β (GSK3B), the homolog of the *Drosophila* gene shaggy. This finding positions APC and GSK3B smack in the center of the wingless signal transduction pathway (Rubinfeld et al., 1996).

TAKE YOUR PARTNER: WHICH WAY β-CATENIN?

β-catenin, freshly released from the ribosome finds three potential binding partners (see Figures 12-6 and 12-7). Two of these, the cadherins and AXIN/APC, are located in the cytoplasm, the other, TCF/LEF, in the nucleus. The first choice is for the cadherins and this association occurs at the exit site of the rough endoplasmic reticulum, though it requires that the cytoplasmic tail of cadherin be phosphorylated. The phosphorylation may occur through casein kinase II-α (CSNK2A1/CSNK2A2) and GSK3B. The attachment of β-catenin then facilitates its transport through the Golgi and prevents degradation of cadherin because it masks the so-called PEST region (or destruction box) that would otherwise be recognized by the Skp-Cullin-F-box (SCF) ubiquitin E3-ligase (Huber et al., 2001). On reaching the membrane, cadherin and β-catenin contribute to the establishment of cell–cell contacts. For more information about ubiquitylation go to Chapter 13, "Activation of the Innate Immune System," Section "Different classes of E3-ubiquitin ligases" (Figure 13-16).

When β-catenin does not bind to cadherins at the rough endoplasmic reticulum, it is directed to its second cytoplasmic partner, the AXIN/APC complex (Su et al., 1993). Here, it is first phosphorylated by casein kinase IIα (CSNK2A1/CSNK2A2) on a ser-45 priming site, which then sets the scene for multiple phosphorylations by GSK3β (GSK3B), on ser-23, -29, -33, -37, and -41. The phosphorylated β-catenin dissociates to undergo multiple rounds of ubiquitylation by the SCF^{BTRC} ubiquitin E3-ligase. Then, being recognized by the regulatory PA700, it is led off

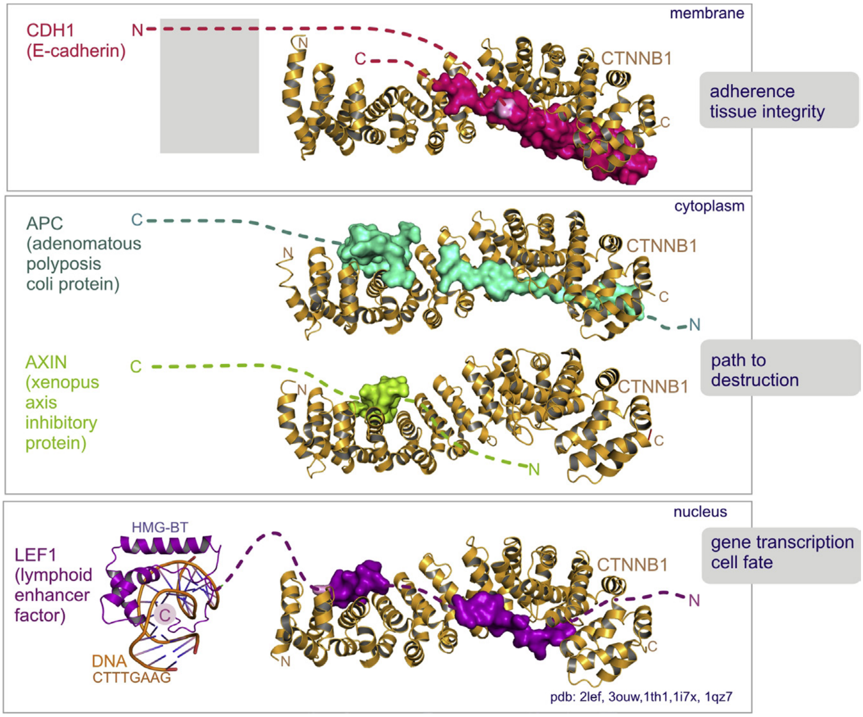


FIGURE 12-7 Take your partner. The protein structures illustrate how the interactions of β-catenin with CDH1, APC, AXIN, and LEF1 all employ the same groove (within the armadillo repeats) and preclude the simultaneous binding of other partners in the region. Effective binding to APC requires prior phosphorylation at T1487, S1498, S1499, S1507, S1510 (not shown).

to destruction in the proteasome (Figure 12-8). Activation of the Wnt pathway allows a stay of execution because it prevents ubiquitylation (Li et al., 2012). The destruction complex saturates with untreated β-catenin and newly synthesized proteins can now enter the nucleus to unite with their third partner, the transcription factors of the TCF/LEF family (in humans principally LEF1 and TCF7) (see below).

The interaction of β-catenin with the different partners occurs through the same groove formed by the armadillo repeats. Binding of one precludes the interaction with another partner. Within the destruction complex, APC seems to play a role in the recruitment of β-catenin. It then passes the protein on to AXIN where it undergoes sequential phosphorylation before returning to APC. From here it must be disposed so that a new β-catenin can enter the process (Xing et al., 2003). AXIN functions as a typically scaffold protein that besides β-catenin, GSK3B, and CSNK2A1/CSNK2A2, interacts with Axam, PP2A, DVL,

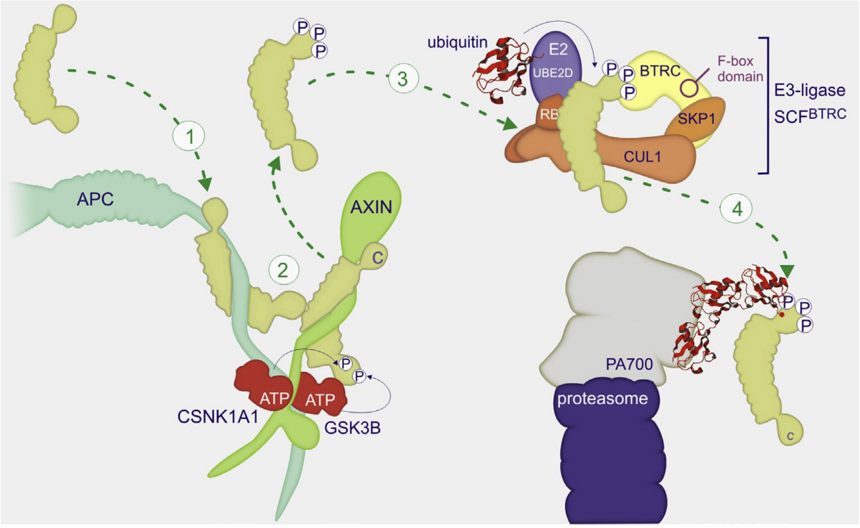


FIGURE 12-8 Path to destruction. Binding of β-catenin to AXIN brings it into the vicinity of CSNK2A1 and GSK3B. These phosphorylate the N-terminal at numerous serines and one threonine residue. When dissociated from the complex, phosphorylated β-catenin is recognized by Trcp1 (BTRC), the receptor component of an SCF^{BTRC} E3-ubiquitin ligase complex (2) The polyubiquitylated protein is next recognized by the PA700 subunit of the proteasome (3) The protein is unfolded and chopped up into small polypeptides.

and LRP5. AXIN not only operates in the WNT pathway but also regulates signaling by transforming growth factor β (TGFβ) by facilitating destruction of the transcription factor SMAD3 (Guo et al., 2008), and it binds to MEKK1 (or -4) and facilitates activation of the JNK pathway (one of the three branches of the MAPK pathways) leading to cell death (apoptosis) (Luo et al., 2003). We deal with TGFβ in Chapter 17, "TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases." For more information about the components of the JNK pathway, return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases" Figure 10-27.

WTN SIGNALING DISABLES THE AXIN–APC DESTRUCTION COMPLEX

Our description of the continuing sequence of events refers to Wnts 1, 3, 3a, and 8. These bind to two receptors, both revealed in genetic screens. The primary binding site constitutes members of the Frizzled family. They are GPCR receptors, spanning the membrane seven times, and having an extracellular cysteine-rich portion (CRD- or Fz-domain)

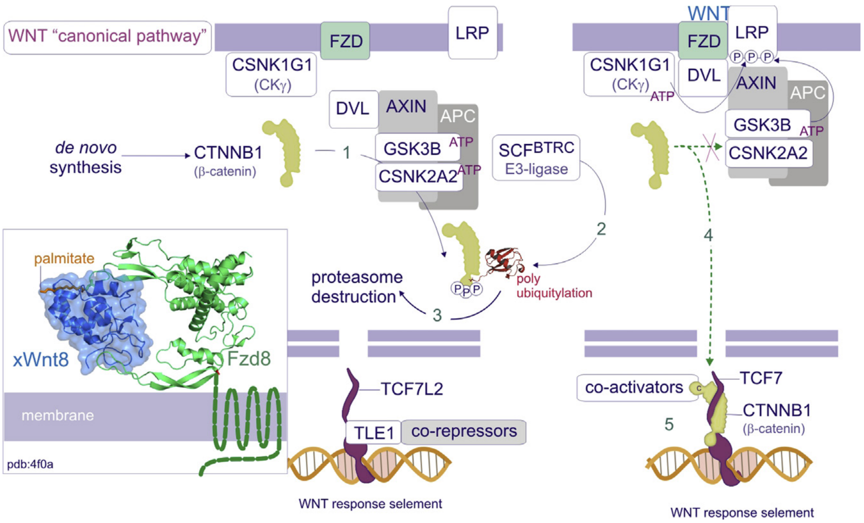


FIGURE 12-9 The canonical Wnt pathway. In the absence of WNT the β-catenin not bound to cadherin is received by the AXIN/APC complex (1) This leads to phosphorylation of the N-terminal first by casein kinase II (dimer of CSNK2A1/-A2) and then by glycogen synthase 3β (GSK3B). The phosphorylated β-catenin is recognized by the receptor moiety of the SCF^{BTRC} ubiquitin E3-ligase leading to the addition of multiple ubiquitins (2) The ubiquitylated β-catenin is then recognized by the proteasome and degraded (3) Binding of WNT to FZD and LRP causes activation of casein kinase 1γ (CSNK1G1). Together with GSK3B, they phosphorylate the cytoplasmic segment of LPR6 at numerous serine and threonine residues allowing AXIN to bind. This results in a functional disabling of the destruction complex and allows accumulation of β-catenin (4) which now enters the nucleus, there to bind to TCF7 (or LEF1). The TCF72L/TLE1 complex is chased from the WNT-response element and replaced by TCF7 (or LEF1) bound to β-catenin. The latter serves as a platform to recruit transcriptional co-activators. The insert shows binding of *Xenopus* Wnt 8 to the Frizzled-8 receptor. Note that Fzd8 offers two binding sites: a hydrophobic pocket for the palmitoyl side chain and the other for a conserved amino acid sequence (like holding a small object between thumb and index finger).

(Bhanot et al., 1996). This group of receptors forms a special class within the GPCRs, named class F (frizzled receptors), counting 11 members (FZD1–10) plus SMO (smoothened). Frizzled offers two binding interactions with WNT, one of them comprising a hydrophobic groove that harbors the palmitoyl side chain (linked to ser-187) and the other through hydrophobic amino acid contacts with the "index finger" segment (insert in Figure 12-9). The WNT–Frizzled interaction is promiscuous because the two binding sites are composed of highly conserved motifs: a single WNT can bind multiple Frizzled proteins and vice versa (Janda et al., 2012). The other receptor (or co-receptor) is the low-density lipoprotein (LDL) receptor-related protein, LRP (the "Arrow" gene in *Drosophila*), which has only a single membrane spanning segment.

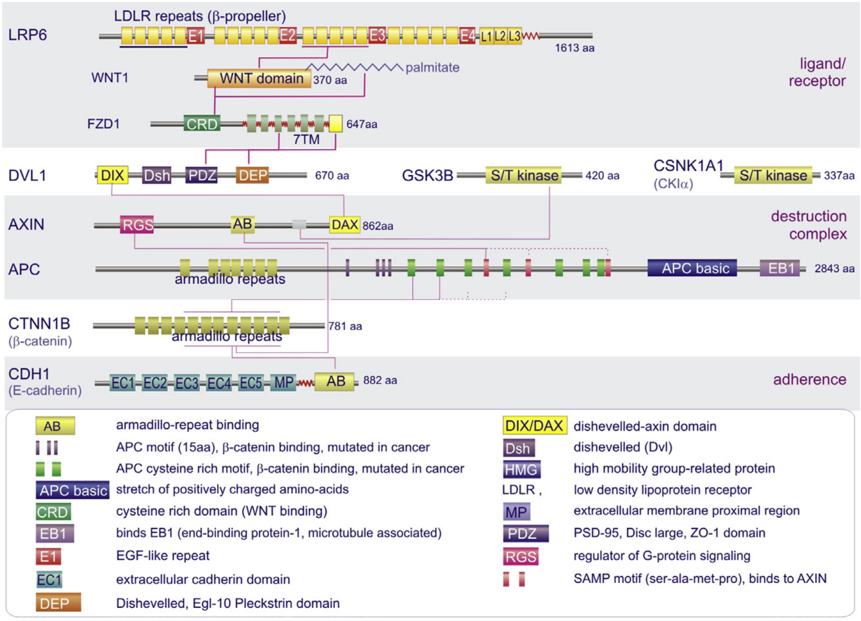

LRP6
LDLR repeats (β-propeller)
E1
E2
E3
E4
L1
L2
L3
1613 aa
WNT1
WNT domain
370 aa
palmitate
ligand/ receptor
FZD1
CRD
647aa
7TM
DVL1
DIX
Dsh
PDZ
DEP
670 aa
GSK3B
S/T kinase
420 aa
CSNK1A1 (CKIα)
S/T kinase
337aa
AXIN
RGS
AB
DAX
862aa
destruction complex
APC
armadillo repeats
APC basic
EB1
2843 aa
CTNN1B (β-catenin)
armadillo repeats
781 aa
CDH1 (E-cadherin)
EC1
EC2
EC3
EC4
EC5
MP
AB
882 aa
adherence
AB armadillo-repeat binding
APC motif (15aa), β-catenin binding, mutated in cancer
APC cysteine rich motif, β-catenin binding, mutated in cancer
APC basic stretch of positively charged amino-acids
CRD cysteine rich domain (WNT binding)
EB1 binds EB1 (end-binding protein-1, microtubule associated)
E1 EGF-like repeat
EC1 extracellular cadherin domain
DEP Dishevelled, Egl-10 Pleckstrin domain
DIX/DAX dishevelled-axin domain
Dsh dishevelled (Dvl)
HMG high mobility group-related protein
LDLR , low density lipoprotein receptor
MP extracellular membrane proximal region
PDZ PSD-95, Disc large, ZO-1 domain
RGS regulator of G-protein signaling
SAMP motif (ser-ala-met-pro), binds to AXIN

FIGURE 12-10 Domain structures of components of the WNT pathway. Domain interactions are shown in pink lines.

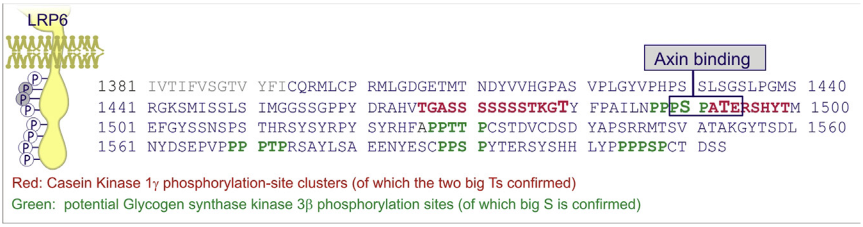
The LRPs also comprise a large family of 11 members of which LRP5 and LRP6 are of particular relevance (Cadigan and Liu, 2005).

The ligand-bound receptor complex causes the recruitment of Disheveled (DVL), activates a protein kinase (CSNK1E1) resulting in phosphorylation of LRP5/6, and then causes recruitment of the above-mentioned destruction complex. The role of Frizzled receptors in all this remains enigmatic. Its cytoplasmic loops bind to DVL (either through an interaction with the PDZ or DEP domain) and this facilitates the subsequent interaction of AXIN with the phosphorylated cytoplasmic segment of LRP. DVL carries a DIX domain that interacts with a similar domain (named DAX) in AXIN (Fiedler et al., 2011) (see Figure 12-10 for domain structures and their interactions). The simultaneous activation of CSNK1E1 and GSK3B leads to multiple phosphorylations of the LRP6 tail (Davidson et al., 2005; Huang et al., 2005). Phosphorylation by CSNK1E1 acts as a primer for the subsequent phosphorylation by GSK3B (in a PPPSPATER motif). How CSNK1E1 is activated is currently not known. The phosphorylated LRP6 tail serves as a second binding site for AXIN (Figure 12-11), now firmly attached to the receptor complex (Mao et al., 2001). Importantly, the attachment of AXIN to the receptor complex disables the destruction complex. A failure to destroy β-catenin (combined with a lack of cytoplasmic binding sites) leads to its nuclear localization. Several modes of transport to the nucleus have been proposed, including free exchange, APC-mediated import, or BCL9-mediated import (protein also known as Legless). BCL9 binds the first (N-terminal) armadillo repeat (Sampietro et al., 2006). In the nucleus, β-catenin finds its third binding partner, the transcription factors of the TCF/LEF-1 family.

Web resources

Learn about the members of the class F GPCR (frizzled type) at the HUGO gene nomenclature committee Web site. URL: http://www.genenames.org/genefamilies/GPCR. For more information about low-density lipoprotein-related proteins (LRPs) URL: http://www.genenames.org/genefamilies/LDLR.

Learn about the genes involved in the Wnt pathway at the Kyoto Encyclopedia of Genes and Genomes database (KEGG map 04310). URL: http://www.kegg.jp/.



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1381 IVTIFVSGTV YFICQRMLCP RMLGDGETMT NDYVVHGPAS VPLGYVPHPS SLSGSLPGMS 1440
1441 RGKSMISSLS IMGGSSGPPY DRAHVTGASS SSSSSTKGTY FPAILNPPPS PATERSHYTM 1500
1501 EFGYSSNSPS THRSYSYRPY SYRHFAPPTT PCSTDVCDSD YAPSRRMTSV ATAKGYTSDL 1560
1561 NYDSEPVPPP PTPRSAYLSA EENYESCPPS PYTERSYSHH LYPPPPSPCT DSS
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Red: Casein Kinase 1γ phosphorylation-site clusters (of which the two big Ts confirmed)

Green: potential Glycogen synthase kinase 3β phosphorylation sites (of which big S is confirmed)

FIGURE 12-11 Numerous casein kinase 1γ and GSK3β phosphorylation sites in the cytosolic sequence of LRP6. LRP6 contains numerous phosphorylation sites. The AXIN-binding site (PPPSPATER) is first phosphorylated on threonine by CK1γ. This acts as a priming site for further phosphorylations by GSK3β. Downstream of the AXIN-binding site, four GSK3β consensus sequences -(S/T)P- are evident (in green).

How exactly the Wnt-receptor complex disables the destruction of β-catenin is not clear. Several propositions exist ranging from: the loss of GSK3B from the destruction complex, the uptake of the destruction complex into early endosomes, followed by the formation of multivesicular endosomes (Taelman et al., 2010), to the model where the destruction complex remains intact but fails to give rise to ubiquitylation of β-catenin (Li et al., 2012). In the latter case it is assumed that phosphorylated β-catenin remains associated with the destruction complex and saturates the binding sites thus allowing newly synthesized protein to escape into the nucleus.

Casein kinases (CSNK): The name casein kinase is misleading because the majority of casein kinases have little or nothing to do with phosphorylation of casein (which is a highly phosphorylated protein present in milk). Casein kinase-2 was discovered in a search for enzymes present in mitochondrial extracts capable of catalyzing the phosphorylation of proteins (Burnett and Kennedy, 1954). In order to test the ability of mitochondrial enzyme systems to phosphorylate proteins other than those native to the organelle, various purified proteins were added to mitochondrial suspensions. Of the proteins tried, only casein showed a large and reproducible phosphorylation. One of its substrates, present in the inner mitochondrial membrane, is glycerol-3-phosphate acyltransferase, involved in de novo glycerolipid biosynthesis (Onorato et al., 2005).

Casein kinases are constitutively active, though not insensitive to allosteric modifications. Access to substrate is controlled by compartmentalization and association of regulatory subunits. Substrates are numerous and include cytoskeletal proteins, receptors, ribosomal proteins, translation and transcription factors, kinases, phosphatases, and metabolic enzymes. There are two main families, CSNK1 and CSNK2, and in addition, a related mammary gland enzyme. The CSNK1 family is further subdivided into CNSK1-A1,-G1, -G2, -G3, -D, and -E. The CSNK1G members are membrane bound because they contain a palmitoylation attachment site. The CSNK2 occurs as a tetramer, comprising two catalytic (CSNK2A1 and CSNK2A2) and two regulatory subunits (CSNK2A3 and CSNK2B). For more information about this constitutively active kinase see Turowec et al. (2010).

REGULATION OF GENE TRANSCRIPTION BY β-CATENIN

In mammalian cells, entry of β-catenin brings about a change in gene expression through an exchange of transcription factors. At the start of the events, parts of the genome are kept in a poorly accessible state through TCF7L1 associated with TLE1 (Groucho). TLE1 forms tetrameric complexes (through intermolecular interactions by their Q-domain) and apart from recruiting histone deacytylase enzymes it also attaches to the tails of histones that constitute the nucleosomes thus condensing the chromatin into polynucleosome arrays (return to Figure 12-5) (Sekiya and Zaret, 2007).

The situation changes with the arrival of WNT. It causes the phosphorylation of TCF7L1 by an as yet unclear signaling event but requiring β-catenin and involving the serine/threonine protein kinase HIPK2 (in *Xenopus*) (Hikasa et al., 2010). Upon phosphorylation, TCF7L1 detaches from DNA and its place is taken by either LEF1 or TCF7. These now bind β-catenin that acts as a platform for the interaction with numerous co-activators. The most important is CREBBP, a histone acetylase that binds the C-terminal of β-catenin and plays a key role in rendering the promoter region accessible to the RNA-polymerase pre-initiation transcription complex. Effective transcriptional activation requires the presence of several other co-activators. These include, BCL9 a protein carrying an HD2-domain that binds the first armadillo repeat. BCL9 then connects with Pygopus (PYGO) and this in turn makes contacts with the mediator complex (a large protein complex that integrates other enhancer elements into the pre-initiation complex). The C-terminal region of β-catenin binds SMARCA4 and SMARCA5 (both components of a large SWI/SNF-related chromatin remodeling complex that functions in an ATPase fashion) and KMT2B and CARM1 (histone-lysine and -arginine methyl transferases, respectively). It also connects directly with MED12 (component of mediator complex) (Figure 12-13). (Tsukiyama, 2002; Hoffmans et al., 2005; Tolwinski and Wieschaus, 2004; Townsley et al., 2004). TCF/β-catenin transcription co-activators are reviewed in Cadigan (2010). Return to Chapter 7, "Bringing the Signal into the Nucleus," Section, "Transcription and transcription factors," Figures 7-10 and 7-11.

MORE ABOUT THE TCF FAMILY

They were initially cloned as a lymphoid cell transcription factor with an HMG domain. Human members of this TCF family are TCF7 (initially TCF1), TCF7L1 (initially TCF3), TCF7L2 (initially TCF4), and LEF1. TCF7L1 and TCF7L2 were named TCF7-like because they came to light in low-stringency hybridization screens with TCF7 cDNA (Korinek et al., 1998a). They all bind β-catenin (through a conserved sequence motif -DExxxFxDEG-), they contain an HMG-box (non-histone high-mobility group nuclear-protein motif) and are recognized as activators and repressors of genes implicated in regulation of the cell cycle in a number of cell types (Figures 12-12 and 12-14). The name T-cell factor should not be confused with the small family of ternary complex factors (TCF, comprising the ELK1, -3, and -4) a quite distinct type of transcription factor linked with the MAP kinases (see Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Section "Beyond ERK"). They should also not be confused with transcription factors 3 and 4 (TCF3 and TCF4), which are members of the basic helix–loop–helix transcription factors.

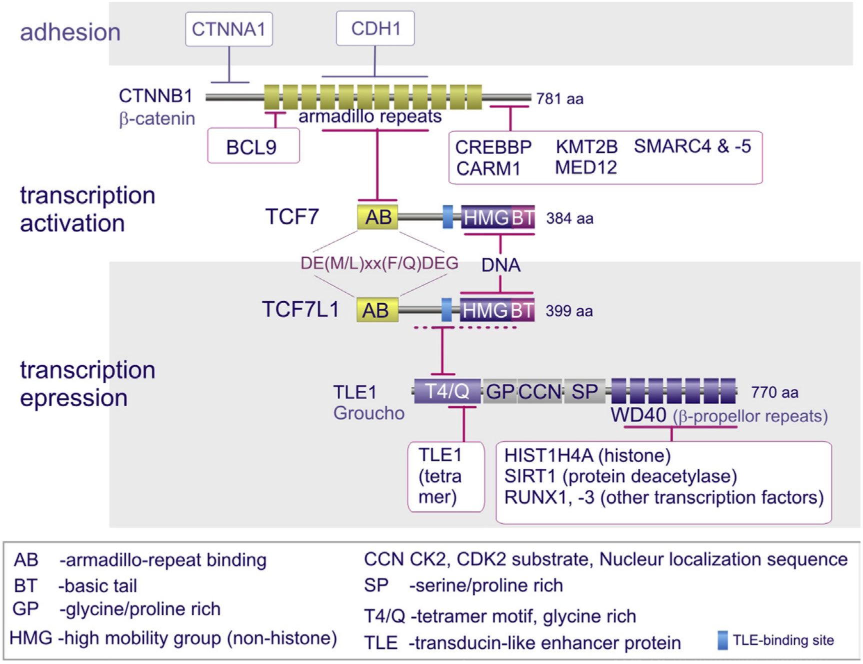

FIGURE 12-12 Interactions of TLE1 and β-catenin (CTNNB1) with components of transcriptional repressor and activator complexes. See Figure 12-13 for more information about their role in the regulation of transcription.

With the exception of TCF7L1, the genes are partly redundant, meaning that you need to knockout at least two in order to obtain an important phenotype. Thus, the knockout of TCF7L1, which is highly expressed in stem cells, suffices to obtain an early embryonic lethal phenotype but for LEF1, TCF7, and TCF7L2 you need combinations of the two. Moreover, they appear to function differently with respect to the recruitment of repressors and activators. TCF7L1 is considered a full-blown repressor, devoid of gene-inducing qualities, whereas LEF1 is the reverse; its function is restricted to activation of WNT target genes and this despite the fact that it can bind co-repressors (like TLE1) (Brantjes et al., 2001). TCF7 and TCFL2 are considered to function in double modes (reviewed in (Cadigan and Waterman, 2012))

The LEF1, TCF7, and TCF7L2 genes possess two promoter sites that give rise to isoforms. In the case of LEF1 and TCF7 a second promotor occurs in the 2nd intron. Transcription thus may give rise to either a full-length protein or a truncated protein, which lacks the armadillo-binding sequence (N-terminal). The truncated proteins act as dominant negative variants because they fail to bind β-catenin and operate in a repression-only mode. Besides a putative second promoter in intron 1,

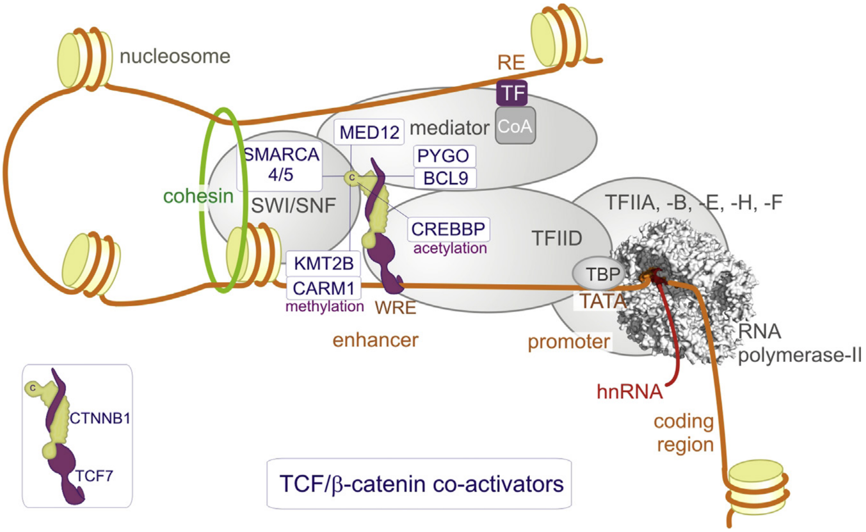

FIGURE 12-13 TCF/β-catenin transcription complex. β-catenin (CTNNB1) bound to TCF7 serves as a platform for the interaction with numerous proteins involved in the regulation of gene transcription. These proteins methylate and acetylate histones, link to the SWI/SNF chromatin remodeling complex (ATP-dependent function), and connect with the mediator complex. Together they make sure that the pre-initiation complex correctly binds the enhancer region and interacts with transcription factors surrounding the promoter region. This drives binding and activity of the RNA-polymerase. WRE, Wnt-response element.

the TCFL2 locus contains a 3rd promoter in intron 5. Both isoforms lack the β-catenin-binding site. Moreover, intron-2 and -4 contain alternative polyadenylation signals. These may lead to premature addition of the poly-A tail to the messenger RNA and result in the production of a C-terminal truncated protein (possessing the armadillo-binding site but lacking the HMG/basic tail domains) (Cadigan and Waterman, 2012). A switch in isoform expression from a WNT-opposing dominant negative to a WNT-promoting full-length isoform of TCF7 has been detected in colon cancer cells (Najdi et al., 2009). The importance of isoform expression is nicely illustrated in mice where the TCF7 gene has been deleted (knockout). They produce adenomas in the intestine because they naturally express the dominant negative isoform, which is protective. In cancer cell lines, which produce the full-length version, loss of expression of TCF7 (through the application of RNAi) results in a reduction of proliferation because they now lack the TCF7 transactivation mechanism (Tang et al., 2008). Likewise, the LEF1 locus is aberrantly activated in colon cancer, but only the promotor for full-length LEF1, the promoter for dominant-negative LEF1 is silenced (Hovanes et al., 2001).

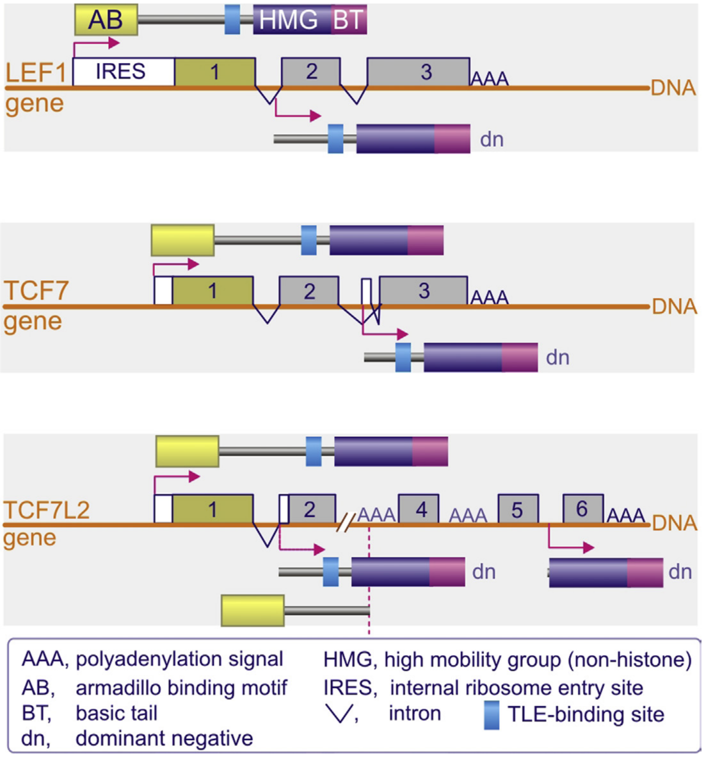

FIGURE 12-14 TCF gene structure and domain architecture. Three of the four genes contain additional initiation sites and these give rise to truncated proteins that lack the armadillo-binding domain (β-catenin-binding site). TCF7L2 has additional poly-A signal motifs which lead to premature polyadenylation of the messenger and gives rise to proteins that lack the DNA-binding segment.

WNT TARGET GENES WITH A WNT-ENHANCER ELEMENT

Many genes are directly affected by Wnt signaling. Here we only consider those having TCF-binding sites. Those listed below should not be understood to be universal targets of Wnt signaling because common transcriptional outputs are unlikely to exist. It is the cell and its context, more than the signal itself, which determines the nature of the response. However, there are some themes that can be discerned in the types of target genes that are induced by WNT:

1. Induction of positive or negative regulators of the signal transduction pathway. Among these are genes encoding TCF7, LEF1, AXIN2, FZD7, WNT3a (mouse), and members of DKK (Dickkopf).

2. Genes involved in proliferation and cell dedifferentiation. Among these are EN2 (engrailed-2), SNAI1, SNAI2 (slug) PPARD, JUN, FOSL (fra1), TCF4 (ITF2), FGF4, FGF18, VEGF, and DPP (*Drosophila*, mammalian homolog of BMP).
3. Genes involved in adhesion, N-cadherin.
4. Genes coding for components of the extracellular matrix and their modifying enzymes, fibronectin, and MMP7.

Many of the WNT-mediated changes in gene transcription resemble those that occur in the epithelial–mesenchymal transition, and the loss of E-cadherin is one of them (see Figure 12-15 and return to Figure 12-2 and Table 12-1) (Christofori, 2003; Pagliarini and Xu, 2003).

Growth factors and WNT work together

The cellular localization of β-catenin determines the fate of mammalian epithelial cells. In the membrane it participates in a complex that gives rise to a polarized epithelial cell having numerous cell-to-cell contacts (return to Chapter 11, "Signal Transduction to and from Adhesion Molecules," Figure 11-20), while in the nucleus it signals a loosely attached cell having a propensity to proliferate and degrade

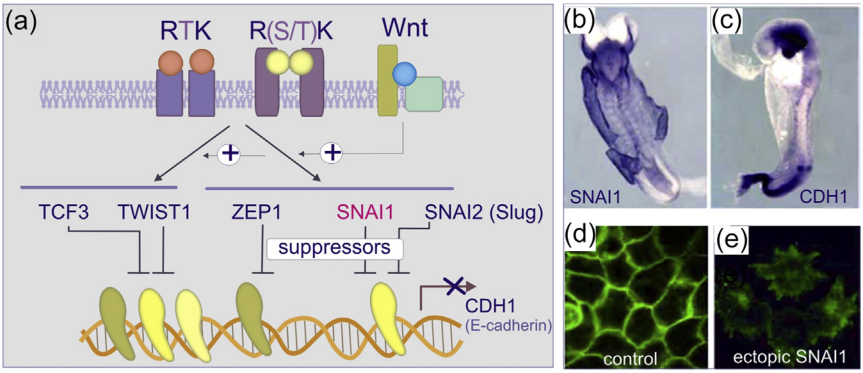

FIGURE 12-15 Supression of E-cadherin expression by growth factors and Wnt. (a) Activation of the receptors for growth factors such as EGF, FGF (RTK), and TGFβ1 (S/T kinases) induces suppressors of E-cadherin expression. The growth factor signals are strongly enhanced by the Wnt signal. (b and c) Expression of the transcription suppressor Snail is inversely related to that of E-cadherin in mouse embryos at 8.5 days of development. Expression of mRNA for E-cadherin or Snail was revealed by an in situ hybridization protocol in which antisense cDNA was coupled to digoxigenin. Hybridized probes were visualized by anti-digoxigenic antibodies coupled to alkaline phosphatase. (d and e) Ectopic expression of snail in epidermal keratinocytes causes an epithelial–mesenchymal transition as indicated by a loss of adherent junctions with loss of epithelial structure and the appearance of detached, nonpolarized cells. Expression of protein was detected by antibodies coupled to a fluorescent dye (Cano et al., 2000).

its extracellular matrix. Although Wnt-mediated effects seem to rely on newly synthesized β-catenin and not on the recycling of the pool of cadherin-associated protein, the stability of the cadherin junctions does, however, play a role. Tyrosine protein kinases involved in "oncogenic transformation" typically fragilize the cadherin core complex. The formation of stable cell–cell adhesions by E-cadherin depends on the association of its cytoplasmic domains with both δ-catenin and β-catenin but also of β-catenin with α-catenin (forming the "core complex") (return to Chapter 11, "Signal Transduction to and from Adhesion Molecules," Figure 11-11(c)). The binding of β-catenin to these partners depends on its phosphorylation status. To maintain this state, receptor tyrosine protein phosphatases, including PTPRM, PTPRB, or PTPRF, and the soluble tyrosine phosphatases PTPN1, ACP1, PTPN11, and PTPRJ, all play an important "protective" role. Opposing effects have been reported for a number of cytokines. The receptors for EGF, FGF, HPG, or TGFβ, directly or indirectly by activation of cytoplasmic tyrosine kinases such as FER, FYN, ABL, or SRC, induce the phosphorylation of cadherin and β-catenin resulting in either uptake of the junctional complex into endosomes or disruption of β-catenin binding (Figure 12-16) (Lilien and Balsamo, 2005).

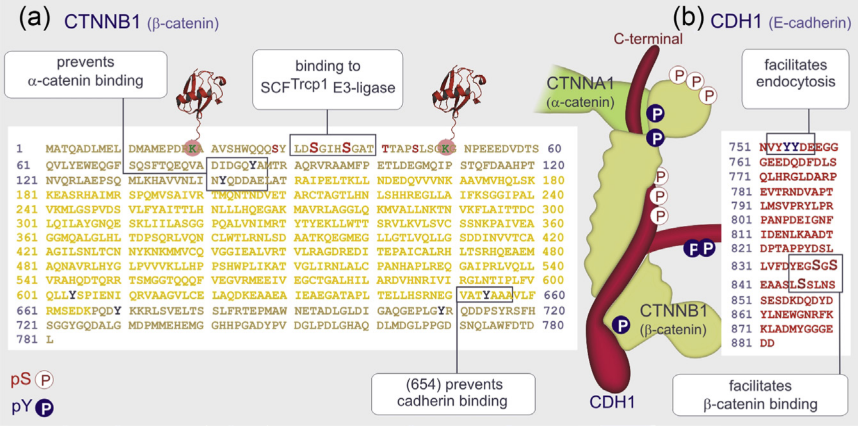
(a) CTNNB1 (β-catenin)
prevents α-catenin binding
binding to SCF^{Trcp1} E3-ligase
1 MATQADLMEL DMAMEPDRKA AVSHWQQQSY LDSGIHSGAT TTAPSLSGKG NPEEEDVDTS 60
61 QVLYEWEQGF SQSFTQEQVA DIDGQYAMTR AQRVRAAMFP ETLDEGMQIP STQFDAAHPT 120
121 NVQRLAEPSQ MLKHAVVNLI NYQDDAELAT RAIPELTKLL NDEDQVVVNK AAVMVHQLSK 180
181 KEASRHAIMR SPQMVSAIVR TMQNTNDVET ARCTAGTLHN LSHHREGLLA IFKSGGIPAL 240
241 VKMLGSPVDS VLFYAITTLH NLLLHQEGAK MAVRLAGGLQ KMVALLNKTN VKFLAITTDC 300
301 LQILAYGNQE SKLIILASGG PQALVNIMRT YTYEKLLWTT SRVLKVLSVC SSNKPAIVEA 360
361 GGMQALGLHL TDPSQRLVQN CLWTLRNLSD AATKQEGMEG LLGTLVQLLG SDDINVVTCA 420
421 AGILSNLTCN NYKNKMMVCQ VGGIEALVRT VLRAGDREDI TEPAICALRH LTSRHQEAEM 480
481 AQNAVRLHYG LPVVVKLLHP PSHWPLIKAT VGLIRNLALC PANHAPLREQ GAIPRLVQLL 540
541 VRAHQDTQRR TSMGGTQQQF VEGVRMEEIV EGCTGALHIL ARDVHNRIVI RGLNTIPLFV 600
601 QLLYSPIENI QRVAAGVLCE LAQDKEAAEA IEAEGATAPL TELLHSRNEG VATYAAAVLF 660
661 RMSEDKPQDY KKRLSVELTS SLFRTEPMAW NETADLGLDI GAQGEPLGYR QDDPSYRSFH 720
721 SGGYGQDALG MDPMMEHEMG GHHPGADYPV DGLPDLGHAQ DLMDGLPPGD SNQLAWFDTD 780
781 L
pS P
pY P
(654) prevents cadherin binding
(b) CDH1 (E-cadherin)
C-terminal
CTNNA1 (α-catenin)
P P P
P
P
P
P
P
P P
CTNNB1 (β-catenin)
P
CDH1
facilitates endocytosis
751 NVYYYDEEGG
761 GEEDQDFDLS
771 QLHRGLDARP
781 EVTRNDVAPT
791 LMSVPRYLPR
801 PANPDEIGNF
811 IDENLKAADT
821 DPTAPPYDSL
831 LVFDYEGSGS
841 EAASLSSLNS
851 SESDKDQDYD
861 YLNEWGNRFK
871 KLADMYGGGE
881 DD
facilitates β-catenin binding

FIGURE 12-16 Determination of cadherin adhesion: phosphorylation and dephosphorylation of the cadherin core complex. Phosphorylation of β-catenin plays an important role in the determination of cell fate. Serine/threonine phosphorylations (indicated in red) prepare the protein to bind to the SCF^{BTRC} E3-ubiquitin ligase and destruction, favoring cell differentiation. The sites of attachment of ubiquitin are highlighted. Serine phosphorylation of E-cadherin facilitates the interaction with β-catenin and promotes formation of adherens junctions. By contrast, phosphorylation on tyrosine residues favors endocytosis of the adhesion complex. Tyrosine phosphorylation of the N-terminal region of β-catenin prevents binding of α-catenin and phosphotyrosines in the last armadillo repeat abrogate binding to E-cadherin. In short, tyrosine phosphorylation destabilizes and this may be one way by which the oncogene SRC facilitates cell transformation (dedifferentiation). Tyrosine phosphorylation sites in blue and serine/threonine in red.

TABLE 12-2 Key determinants of cell fate

Differentiation	
Destruction of β-catenin	S/T phosphorylation by CKI (Ser-45 primer) and GSK3b (other sites)
Stabilization of E-cadherin core complex	serine phosphorylation on E-cadherin by CKII and GSK3b
Stabilization of E-cadherin core complex	Dephosphorylation of β-catenin by tyrosine phosphatases (e.g., PTP1B, LMW-PTB, SHP2 and DEP (cytosol) and VEPTP, LAR, or PTPμ (transmembrane)
Dedifferentiation	
Destabilization of cadherin core complex	In response to EGF, FGF, HGF/SF, or TGFb, tyrosine phosphorylation by Src, Fer, Fyn, abl, or c-met
Endocytosis of E-cadherin core complex	Tyrosine phosphorylation on residues 754–755

Moreover, phosphorylation of β-catenin by the EGFR increases binding to the TATA box-binding protein (TBP), whereas phosphorylation by FER, FYN, or MET promotes binding to BCL9. Either way these phosphorylations favor nuclear localization of β-catenin. Not only this, but FGF, EGF, and TGFβ also cooperate in the expression of numerous transcription factors (TCF3, TWIST, ZEB1, SNAI1, and SNAI2 (Slug)) that repress transcription of E-cadherin (return to Figure 12-15). Dedifferentiation is favored by tyrosine phosphorylations of β-catenin and E-cadherin. These destabilize the cadherin core complex by dissociating β-catenin from E-cadherin. Of note is β-catenin phosphorylation on residue Y654 by SRC, a known oncogene. The aforementioned growth factors also induce the pro-migratory adhesion molecule N-cadherin. The key determinants of cell fate are summarized in Table 12-2.

EXTRACELLULAR INHIBITORS OF WNT AND ITS RECEPTORS

A number of proteins regulate Wnt signaling negatively. They do this by binding either to WNT itself or to the WNT receptors. Dickkopf (DKK), one of the best characterized, down-regulates the activity of the LRP5/6 receptor. Dickkopf are secreted proteins, four members in human and mouse. Binding to LRP5/6 requires the presence of a member of KREMEN, a cell surface protein (kringle-containing protein marking the eye and the nose). Thus DKK2 binds to LRP6 and KREMEN2 in order to prevent WNT binding and thus inhibiting Wnt signaling. In *Xenopus laevis* embryos they prevent dorsalization (Mao and Niehrs, 2003; Ahn et al., 2011). Another inhibitor is sclerostin (SOST), that binds LRP6 and prevents WNT8 binding (Itasaki et al., 2003). Patients with distinct types of hereditary high bone mass diseases were found to carry mutations in the LRP5 extracellular domain, which render LRP5 resistant to binding of the antagonist SOST and DKK1. Similarly, loss-of-function mutations (protein retained in endoplasmic reticulum) in the SOST gene cause sclerosteosis (reviewed in (Clevers and Nusse, 2012)). Another example is WIF1, highly expressed in the unsegmented paraxial presomitic mesoderm in *X. laevis* embryos (Hsieh et al., 1999). Also, there are the secreted Frizzled receptor-related proteins (SFRP), five members in human that sequester WNT (Finch et al., 1997). As a final example, APCDD1 is a membrane-bound glycoprotein that inhibits Wnt signaling by binding to both WNT and LRP. It is mutated in hereditary hypotrichosis simplex, a condition characterized by hair follicle miniaturization (Shimomura et al., 2010). None of these proteins exist in *Drosophila*.

CONTRIBUTION OF DIFFERENT SPECIES TO THE ELUCIDATION OF THE WNT SIGNAL TRANSDUCTION PATHWAY

We have limited the description of the discovery of Wnt and its downstream signaling components to the epidermal segment polarity of the larval cuticle in *Drosophila*. However, experiments using *Xenopus laevis* have also contributed to the elucidation of this pathway. The *Xenopus* model attracted much attention after it was found that injection of Wnt mRNA in developing embryos causes a new and complete dorsal–ventral axis, giving rise to two-headed tadpoles (Sokol et al., 1991). The local production of Wnt creates an independent Spemann organizer. With respect to molecular mechanisms of Wnt signaling, *Xenopus* has helped to elucidate the mechanism of β-catenin stabilization and subsequent nuclear localization, it has helped to define the Wnt co-receptors LRP5 and LRP6, the discovery that β-catenin binds TCF (XTcf-3) (Moolenaar et al., 1996) and the Wnt antagonists such as DKK, sFRP, and Cerberus. Finally, *Xenopus* has played an important role in the discovery of the switch action of TCF, being instrumental both in repression and activation (Fan et al., 1998) and in the elucidation of non-canonical (β-catenin-independent) pathways. For a review, see Moon (Moon et al., 1998). For more information on the Spemann organizer we refer to Chapter 17, "TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases" Section "SMAD Proteins as Integrators of Signal Pathways."

WNT SIGNALING AND STEM CELL SELF-RENEWAL

The stem cell niche

The absorptive epithelium of the small intestine is ordered into villi and the crypts of Lieberkühn (Figure 12-17). Differentiated cells that reside on the villi are mainly enterocytes, whose role is to transport metabolites toward the interior. They are interspersed by a few enteroendocrine cells, which secrete into the blood the hormones gastrin, secretin, and cholecystokinin in response to the intestinal content, and

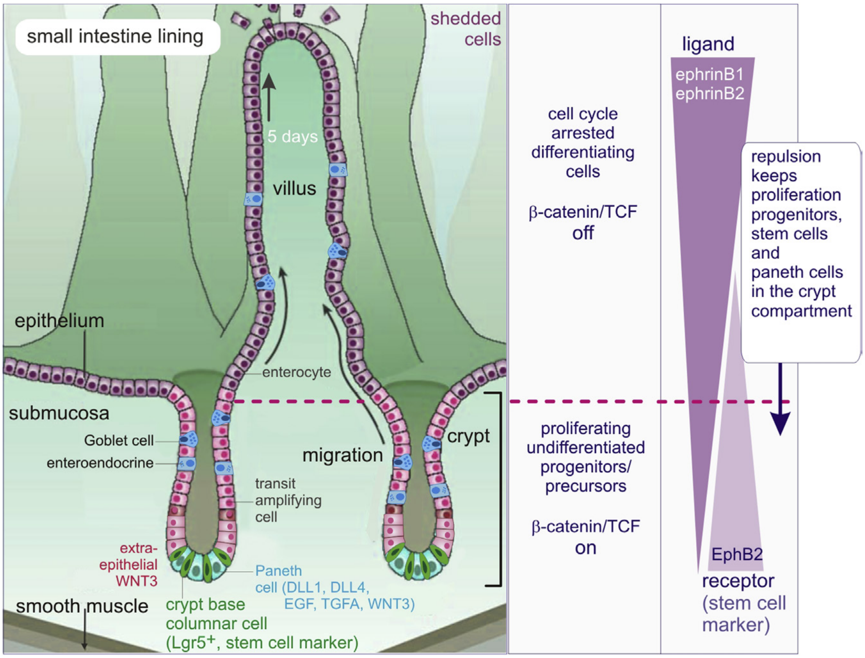
small intestine lining
shedded cells
5 days
villus
epithelium
enterocyte
submucosa
Goblet cell
enteroendocrine
migration
crypt
transit amplifying cell
extra-epithelial WNT3
Paneth cell (DLL1, DLL4, EGF, TGFA, WNT3)
smooth muscle
crypt base columnar cell (Lgr5+, stem cell marker)
cell cycle arrested differentiating cells
β-catenin/TCF off
proliferating undifferentiated progenitors/ precursors
β-catenin/TCF on
ligand
ephrinB1
ephrinB2
repulsion keeps proliferation progenitors, stem cells and paneth cells in the crypt compartment
EphB2
receptor
(stem cell marker)

FIGURE 12-17 Epithelium of the small intestine. Left panel: The crypts of the small intestine contain stem cells that continuously produce committed progenitors (transit amplifying cells). These either remain in the crypt to transform into Paneth cells or they move up the villi to form the absorptive epithelium (left panel). Paneth cells produce cytokines essential for the maintenance of the stem cell niche. WNT3 is produced both by Paneth and by extra-epithelial cells (perhaps surrounding fibroblasts). Right panel: Wnt contributes to the maintenance of the stem cell niche by assuring the proliferation and self-renewal of the stem cells (but not the committed progenitors) and also their localization within the crypt. It does this by induction of the ephrin receptor B2 and by inhibiting expression of the ligands ephrin-B1 and -B2. This creates opposing gradients of both receptor and ligand. Stem cells and progenitors expressing high levels of the receptor are prevented from migrating out of the crypt through the repulsive action of the ephrins. Only when committed progenitor cells have switched off the canonical Wnt pathway do they escape repulsion and move up to the villus (Sancho et al., 2004).

its acidity, and goblet cells, which secrete the mucin that lubricates the gut lining. Paneth cells, which reside in the crypts, are functionally similar to neutrophils and contribute to the extrinsic gastrointestinal barrier by releasing α-defensins (cryptdins), lysosyme, and phospholipase A2. The crypts also harbor the stem and the committed progenitor-cell compartment (transit-amplifying cells), situated halfway between the bottom of the crypt and the start of the villi. The colon epithelium is different in that it has a flat surface (no villi) pitted with crypts. The upper segment of the crypt contains goblet cells and mature enterocytes, whereas the lower segment is dominated by the stem cell/progenitor cell compartment.

The lifespan of intestinal epithelial cells is 4–5 days, during which time they migrate from the base of the crypts to the villous tips (Cheng and Leblond, 1974). Here they die and are shed into the fecal stream (Figure 12-17). Stem cells (as we will see later these are Lgr5+ basal crypt columnar cells) and Paneth cells escape migration (see “Wnt aligns committed progenitor cells along the crypt/villus axis”). The stem cells cycle slowly, one division per day, continuously producing (committed) progenitor cells that proliferate more rapidly (a total of four to five times). As they attain the crypt–villous axes in the small intestine (or the upper crypt segments in the colon), they undergo cell cycle arrest and commence expressing differentiation marker (Booth and Potten, 2000). The proliferation and the acquisition of particular cell fates is coordinated by a small number of highly evolutionarily conserved signaling pathways, including the Wnt/β-catenin and the Notch signaling pathways. The crypt epithelium exhibits nuclear β-catenin, which is, of course, a hallmark of Wnt signaling.

The canonical Wnt-signal pathway is vital for the maintenance of the epithelial stem cell niche. Deletion of TCF7L2 blocks the development of the intestinal crypts in mice (Korinek et al., 1998b). Its functional loss in adult animals (with established crypts), obtained by expression of the Wnt inhibitor Dickkopf 1 (DKK1) or by deletion of the genes encoding for β-catenin (Fevr et al., 2007) or TCF7 (van Es et al., 2012), results in the loss of proliferation of the crypt cells (i.e., loss of stem cell function). In exploring the Wnt-driven genetic program, one of the TCF7 target genes (of a list of 80 or so genes), LGR5, was expressed in a unique fashion, limited to a few small cells interspersed by the Paneth cells. LGR5 qualifies as an orphan leucine-rich repeat-containing G-protein-coupled receptor. The $Lgr5^+$ cells had already been identified histologically, back in 1974, as “crypt base columnar cells” and were tentatively earmarked as intestinal stem cells (Cheng and Leblond, 1974). Highly sophisticated morphological and marker-expression studies revealed that, indeed, all differentiated cell types in the intestinal epithelium originated from the $Lgr5^+$ crypt base columnar cells (Figure 12-17). Lineage tracing persisted over the lifetime of experimental animals, thus demonstrating that Lgr5$^+$ stem cells are long lived and multipotent stem cells (giving rise to multiple cell lineages) (Barker et al., 2007).

The intestinal stem cells divide symmetrically such that every stem cell division gives rise to two equipotent daughters. Yet, in general, only one of the daughters remains stem cell and the other becomes a committed progenitor (transit amplifying cell). The fate is sealed by the neighboring Paneth cell; you make contact and remain stem cell, or you do not and you turn into a committed progenitor. This model of "neutral competition" (that is each daughter has an equal chance to remain stem cell) implies that stem cell longevity is an attribute of a cell population, not of a set of "elected stem cells." To maintain the stem cell condition of Lgr5$^+$ cells, Paneth cells provide the membrane-bound ligands DLL1 and DLL4, which feed the NOTCH signal pathway (juxtacrine signal) and they produce EGF, TGFA, and WNT3 (paracrine signal) (Figure 12-18) (Sato et al., 2011). We return to NOTCH in Chapter 19, "Cell Fate Determination by Notch." How exactly WNT3 contributes to the maintenance of the stemness of Lgr5+ cells is not known but we can evoke the current concept that stem cells are intrinsically destined to differentiate, and stem cells factors therefore generally play an inhibitory role (allowing master transcription factors to keep much of the DNA accessible).

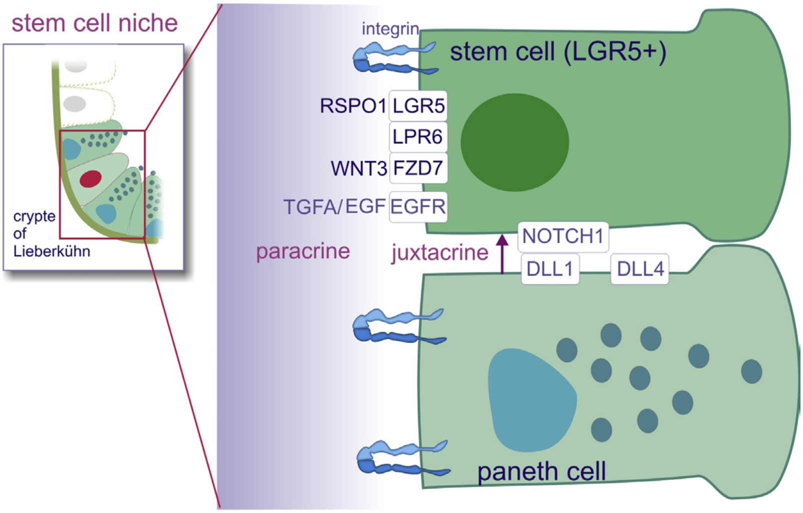

FIGURE 12-18 Paneth cells play a role in maintaining the stem cell niche. Through the release of cytokines, EGF, TGFA, and WNT3, and the membrane expression of the DLL1, DLL4 (ligands for the NOTCH receptor), Paneth cells hold their neighboring cell in a stem cell condition. Of these cytokines, WNT3 is also produced by an extra-epithelial source. RSPO1, of unknown source, binds to LGR5 and renders the Wnt response much more efficient.

R-spondins potentiate the Wnt signal

Efficient stem cell culture requires the presence of R-spondin 1 (RPSO1), a secreted protein first observed in the neural tube at the boundary between the roof plate and the neuroepithelium (Kamata et al., 2004). It contains a thrombospondin type-I repeat hence the name spondin. It is part of a family of four members (RPSO1–4). They all bind to LGR5, one of the markers of the intestinal stem cell, and they potentiate the action of low concentrations of WNT (Kazankava et al., 2004). Deletion of LGR5 brings about a change in the gene program that corresponds with genes activated by the Wnt pathway and it was concluded that RPSO proteins boost the canonical pathway (Schuijers and Clevers, 2012). Immunoprecipitation studies revealed that LGR4 and -5 coprecipitate with FZD5 and -7 and with LRP5 and -6; they form signaling complexes. LGR proteins share the characteristics of G-protein-coupled receptors and they classify among the class A "rhodopsin" receptors that lack ligand (class 17, "orphan receptors") in the HUGO gene classification. They bear most resemblance with the LH, TSH, and FSH receptors. For more information about GPCR proteins, return to chapter 5, "Sensory Signal Processing," Figure 5-22. However, until today no G-protein-associated signaling events have been reported. So how LGR5 enhances the WNT signal is still unclear. As already mentioned, LGR5 is one of targets of WNT and it operates in a positive feedback circuit, reinforcing the Wnt signal. Lgr5$^+$ stem cells occur in other epithelia such as the one of the colon, stomach, hair follicles (Alonso and Fuchs, 2003), and mammary glands. They are typical of epithelia with high turnover and unidirectional displacement of committed progenitor cells. These stem cells all depend on WNT and tumors arising from these cells often carry mutations in the Wnt pathway. Finally, hematopoietic stem cells too require the action of WNT (Reya et al., 2003; Willert et al., 2003).

Wnt aligns committed progenitor cells along the crypt/villus axis

A screen for genes either induced or repressed by β-catenin/TCF7L2 in intestinal epithelial cells revealed both Ephrin-B receptors (EphB) and their ligands (B-type ephrins) among the targets (van de Wetering et al., 2002). Here, β-catenin/TCF exerts inverse control, enhancing expression of the receptors and suppressing the ligands. This creates opposed gradients, with high receptor expression at the base of the crypts and high ligand expression at the points of the villi (return to Figure 12-17).

Expression of EphB receptors is essential for the correct positioning of epithelial cells along the crypt/villous axis. Although they maintain their intestinal villi, mice carrying EphB2/EphB3 double mutants lose the typical localization of the Paneth cells in the crypts and the typical compartmentation of proliferating and differentiated cells along the axis (put simply, everything is higgledy-piggledy) (Battle et al., 2002). EphB receptors, which are highly expressed at the bottom of the crypt, may thus act to restrict the upward migration of stem and Paneth cells.

Ephrin receptors represent the largest subfamily of receptor tyrosine kinases (14 members in human). Based on their ligand-binding specificities, these receptors are grouped into two subclasses: EphA receptors bind A-type ephrins and EphB bind B-type ephrins. Ephrin ligands are GPI-anchored proteins and the interaction between Eph receptors and their ligands therefore involves direct cell–cell interactions. Eph receptors and ligands are said to be involved in directed migration through repulsion. They constitute repulsive clues in processes such as axon pathfinding, migration of neural crest cells, and boundary formation between segmented structures such as rhombomeres (Wilkinson, 2001).

WNT AND PLANAR CELL POLARITY

Disheveled and frizzled have been key phenotypes in the unraveling of the Wnt pathway. The disorder in wing hair orientation is due to failure in establishing uniform PCP. Wingles (Wnt) coordinates the nucleation and organization of actin filaments that eventually give rise to actin bundles that create the hair. The pathway does not involve β-catenin and qualifies as a noncanonical pathway (return to Figure 12-1). The GTPase Rho1 is a key regulator in the organization of the actin cytoskeleton (Hall and Nobes, 2000) and it acts downstream of Frizzled/Disheveled. Rho1 interacts with the effector diaphanous (dia) which is connected with profilin (chic), a protein involved in the process of actin polymerization. Through another effector, Rho coiled-coil kinase (dRok), Rho1 converts these filaments into contractile fibers, combining them with actinin and activating the motor protein myosin-II. A similar pathway exists in mammalian cells (Figure 12-19) (Fanto and McNeill, 2004).

Both dRok and dia also operate in centrosome positioning during prometaphase (Narumiya and Yasuda, 2006). They control the connection of the astral microtubules with the cell cortex and hence they determine the orientation of the mitotic spindle (Cowan and Hyman, 2004). Interestingly, APC, a component of the β-catenin destruction complex, also binds microtubules and is involved in organizing their linkage to the cortical protein network (return to Chapter 9, "Protein Kinase C in Oncogenic Transformation and Cell Polarity") (Figures 9-14 and 9-22) (Reilein and Nelson, 2005). Indeed Wingles (Wnt) also plays a role in spindle orientation in sensory organ precursor cells. It is important to note that Wingless operates amidst other factors. For instance Stan (fmi) and Vang (Stbm) plays an essential inhibitory role at the proximal end of the epithelial cell (121) Strutt and Warrington, 2008. Other polarity cues may be involved as well (122) Sagner et al., 2012.

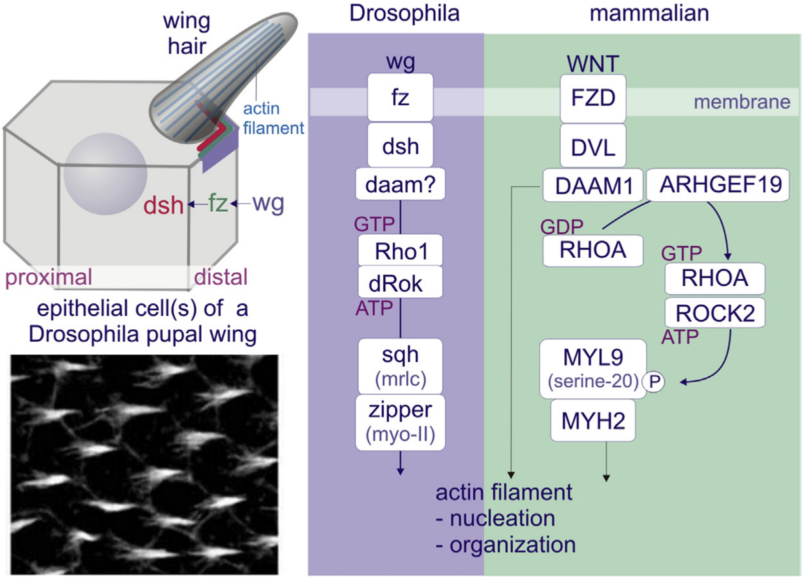

FIGURE 12-19 Planar cell polarity. Polarization of Wnt, Fz, and Dsh causes the distal organization of the actin cytoskeleton and this gives rise to the formation of a distal hair on the wings of *Drosophila*. Loss of Wnt signaling results in a more random position of the hairs and this gives rise to the frizzled and disheveled phenotype (see also Figure 12-3). *Scanning electron microscopy image of pupal wing hairs courtesy of Dr David Strutt, Sheffield University, UK.*

MUTATIONS OF CTNNB1, AXIN, AND APC IN HUMAN CANCERS

Several mutations in components of the Wnt signal transduction pathway give rise to cancer (Lynch and de la Chapelle, 2003; Clevers and Nusse, 2012). Most of these result in the expression of nonfunctional proteins at both alleles. A prime example is the APC gene. Here, the majority of mutations accumulate in the so-called mutation cluster region in the central part of the peptide chain (return to Figure 12-10, "APC motifs"). The truncated proteins that arise are unable to bind β-catenin (CTNN1B) and AXIN (Miyoshi et al., 1992). Cancer-generating mutations also occur in AXIN and in β-catenin (Morin et al., 1997). In gain-of-function mutations, β-catenin is protected against degradation because the mutations either replace the phosphorylation sites for CNSK1A1 or GSK3B in the destruction box or they prevent its binding to the AXIN/APC complex. They are therefore considered as "activating" mutations (even though they do not actually activate the protein). In some patients having familial gastric cancer, the gene for E-cadherin (CDH1) expresses a truncated product (Guilford et al., 1998), which is unable to participate in junctional complexes. As a result, the adherent junctions are improperly formed so that over time, the cells detach and become invasive.

DIFFERENT TYPES OF MUTATIONS IN APC

The penetrance of germ-line mutations that increase the risk of colorectal cancer varies. The APC polymorphism i1307 K has a penetrance of 20% meaning that one in five persons that carry it will develop APC. This mutation does not directly affect the activity of APC but enhances the chance of further "somatic" mutations. The reason for this is that in the mutated protein, the codon ATA (isoleucine) has been changed to AAA (leucine). This gives rise to a DNA sequence consisting of eight adenosines in a row and which increases the risk of slippage during replication. It therefore increases the chance of further mutations of which some will eventually give rise to a frame shift and loss of APC function. Truncation mutations have a penetrance that approach 100%.

Several somatic mutations in β-catenin have been associated with colorectal, liver, and hair follicle cancers. These mutations often occur at sites subject to phosphorylation by CNSK1A1 and GSK3B. Mutations are also found in armadillo repeats: mutations in the 3rd and 4th repeat affect binding to APC or AXIN, mutations in the 12th enhance transcriptional activity. All these are so-called "activation mutations" because they enhance the output of β-catenin, either through an increase in protein content or by an increase in its transcriptional activity.

Web resource

For more information about the involvement of mutations of β-catenin (CTNNB1) in disease we propose to consult the OMIM database. Pertinent entry numbers are: 116806 (information about CTNNB1), 114500 (colorectal cancer), 132600 (pilomatrixoma), 155255 (medulloblastoma), 167000 (ovarian cancer), and 615075 (mental retardation). URL: http://www.omim.org/.

References

van Amerongen, R., Nusse, R., 2009. Towards an integrated view of Wnt signaling in development. Development 136, 3205–3214.

Ahmed, Y., Hayashi, S., Levine, A., Wieschaus, E., 1998. Regulation of armadillo by a *Drosophila* APC inhibits neuronal apoptosis during retinal development. Cell 93, 1171–1182.

Ahn, V.E., Chu, M.L., Choi, H.-J., Tran, D., Abo, A., Weis, W.I., 2011. Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6. Dev. Cell 21, 862–873.

Alonso, L., Fuchs, E., 2003. Stem cells in the skin: waste not, Wnt not. Genes Dev. 17, 1189–1200.

Baron, R., Kneissel, M., 2013. Wnt signaling in bone homeostasis and disease: from human mutations to treatments. Nat. Med. 19, 179–192.

Berx, G., van Roy, F., 2001. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res. 3, 289–293.

Baker, N.E., 1987. Molecular cloning of sequences from wingless, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. EMBO J. 6, 1765–1773.

Brunner, E., Peter, O., Schweizer, L., 1997. Pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. Nature 385, 829–833.

Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., Birchmeier, W., 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382, 638–642.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., Nusse, R., 1996. A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature 382, 225–230.

Burnett, G., Kennedy, E.P., 1954. The enzymatic phosphoryation of proteins. J. Biol. Chem. 211, 969–980.

Brantjes, H., Roose, J., van de Wetering, M., Clevers, H., 2001. All TCF HMG box transcription factors interact with Groucho-related co-repressors. Nucleic Acids Res. 29, 1410–1419.

Booth, C., Potten, C.S., 2000. Gut instincts: thoughts on intestinal epithelial stem cells. J. Clin. Invest. 105, 1493–1499.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., Clevers, H., 2007. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. Nature 449, 1003–1007.

Battle, E., Henderson, J.T., van den Born, M.M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., Clevers, H., 2002. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 111, 251–263.

Cabrera, C.V., Alonso, M.C., Johnston, P., Phillips, R.G., Lawrence, P.A., 1987. Phenocopies induced with antisense RNA identify the wingless gene. Cell 50, 659–663.

Clevers, H., van de Wetering, M., 1997. TCF/LEF factor earn their wings. Trends Genet. 13, 485–489.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., Bejsovec, A., 1998. *Drosophila* Tcf and Groucho interact to repress Wingless signaling activity. Nature 395, 604–608.

Cadigan, K.M., Liu, Y.I., 2005. Wnt signaling: complexity at the surface. J. Cell Sci. 119, 395–402.

Cadigan, K.M., 2010. TCFs and WNT/β-catening signaling: more than one way to throw the switch. Curr. Top. Dev. Biol. 98, 1–34.

Cadigan, K.M., Waterman, M.L., 2012. TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb. Perspect. Biol. 4, a007906.

Christofori, G., 2003. Changing neighbours, changing behaviour: cell adhesion molecule-mediated signaling during tumour progression. EMBO J. 22, 2318–2323.

Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., Nieto, M.A., 2000. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. 2, 76–83.

Clevers, H., Nusse, R., 2012. Wnt/β-Catenin signaling and disease. Cell 149, 1192–1205.

Cheng, H., Leblond, C.P., 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. Columnar cell. Am. J. Anat. 141, 461–479.

Cowan, C.R., Hyman, A.A., 2004. Asymmetric cell division in *C. elegans*: cortical polarity and spindle positioning. Annu. Rev. Cell Dev. Biol. 20, 427–453.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A., Niehrs, C., 2005. Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. Nature 438, 867–872.

Eger, A., Stockinger, A., Schaffhauser, B., Beug, H., Foisner, R., 2000. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J. Cell Biol. 148, 173–188.

van Es, J.H., Haegebarth, A., Kujala, P., Itzkovitz, S., Koo, B.K., Boj, S.F., Korving, J., van den Born, M., van Oudenaarden, A., Robine, S., Clevers, H., 2012. A critical role for the wnt effector tcf4 in adult intestinal homeostatic self-renewal. Mol. Cell Biol. 32, 1918–1927.

Fialka, I., Schwarz, H., Reichmann, E., Oft, M., Busslinger, M., Beug, H., 1996. The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. J. Cell Biol. 132, 1115–1132.

Franch-Marro, X., Wendler, F., Griffith, J., Maurice, M.M., Vincent, J.P., 2008. In vivo role of lipid adducts on Wingless. J. Cell Sci. 121, 1587–1592.

Fiedler, M., Mendoza-Topaz, C., Rutherford, T.J., Mieszczanek, J., Bienz, M., 2011. Dishevelled interacts with the DIX domain polymerization interface of Axin to interfere with its function in down-regulating β-catenin. PNAS U.S.A. 108, 1937–1942.

Finch, P.W., He, X., Kelley, M.J., 1997. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. PNAS U.S.A. 94, 6770–6775.

Fan, M.J., Gruning, W., Walz, G., Sokol, S.Y., 1998. Wnt signaling and transcriptional control of Siamois in Xenopus embryos. PNAS U.S.A. 95, 5626–5631.

Fevr, T., Robine, S., Louvard, D., Huelsken, J., 2007. Wnt/beta–catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol. Cell Biol. 27, 7551–7559.

Fanto, M., McNeill, H., 2004. Planar polarity from flies to vertebrates. J. Cell Sci. 117, 527–533.

Gottardi, C.J., Wong, E., Gumbiner, B.M., 2001. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. J. Cell Biol. 153, 1049–1060.

Gerhart, J., 1999. Signaling pathways in development. Teratology 60, 226–239.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., et al., 1991. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 66, 589–600.

Guo, X., Ramirez, A., Waddell, D.S., Li, Z., Liu, W., Wang, X.F., 2008. Axin and GSK3 control Smad3 protein stability and modulate TGF-sginaling. Genes Dev. 22, 106–120.

Guilford, P., Hopins, J., Harraway, J., et al., 1998. E-cadherin germline mutations in familial gastric cancer. Nature 392, 402–405.

Hay, E.D., 2005. The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Dev. Dyn. 33, 706–720.

Huber, M.A., Kraut, N., Beug, H., 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr. Opin. Cell Biol. 17, 548–558.

Hinck, L., Nelson, W.J., Papkoff, J., 1994. Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin. J. Cell Biol. 124, 729–741.

Huber, A.H., Stewart, D.B., Laurents, D.V., Nelson, W.J., Weis, W.I., 2001. The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover. J. Biol. Chem. 276, 12301–12309.

Huang, H., Tamai, K., Doble, B., Li, S., Habas, R., Okamura, H., Woodgett, J., He, X., 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438, 873–877.

Hikasa, H., Ezan, J., Itoh, K., Li, X., Klymkowsky, M.W., Sokol, S.Y., 2010. Regulation of TCF3 by Wnt-dependent phosphorylation during vertebrate axis specification. Dev. Cell 19, 521–532.

Hoffmans, R., Stadeli, R., Basler, K., 2005. Pygopus and legless provide essential transcriptional coactivator functions to armadillo/beta-catenin. Curr. Biol. 15, 1207–1211.

Hovanes, K., Li, T.W., Munguia, J.E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R.F., Waterman, M.L., 2001. β-catenin-sensitive isoforms of mylphoid enhancer faoactor-A are selectively expressed in colon cancer. Nat. Genet. 28, 53–57.

Hsieh, J.C., Kodjabachian, L., Rebbert, M.L., Rattner, A., Smallwood, P.M., Samos, C.H., Nusse, R., Dawid, I.B., Nathans, J., 1999. A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398, 431–436.

Hall, A., Nobes, C.D., 2000. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355, 965–970.

Itasaki, N., Jones, C.M., Mercurio, S., et al., 2003. Wise, a context-dependent activator and inhibitor of Wnt signaling. Development 130, 4295–4305.

Janda, C., Waghray, D., Levin, A., Thomas, C., Garcia, K., 2012. Structural basis of Wnt recognition by Frizzled. Science 337, 59–64.

Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N., Budnik, V., 2009. Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. Cell 139, 393–404.

Klingensmith, J., Nusse, R., 1994. Signaling by wingless in *Drosophila*. Dev. Biol. 166, 396–414.

Korinek, V., Barker, N., Willer, K., Moolenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O., Clevers, H., 1998a. Two members of the TCF family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. Mol. Cell Biol. 18, 1248–1256.

Korinek, V., Barker, N., Moerer, P., Van Donselaar, E., Huls, G., Peters, P.J., Clevers, H., 1998b. Depletion of epithelial stem–cell compartments in the small intestine of mice lacking Tcf–4. Nat. Genet. 19, 379–383.

Kamata, T., Katsube, K., Michikawa, M., Yamada, M., Takada, S., Mizusawa, H., 2004. R-spondin, a novel gene with thrombospondin type-1 domain, was expressed in the dosar neuroal tube and affected in Wnts mutants. Biochim. Biophys. Acta 1676, 51–62.

Kazankava, O., Glinka, A., del Barco Barrantes, I., Stannek, P., Niehrs, C., Wu, W., 2004. R-spondin2 is a secreted activator of Wnt/β-catenin signalin and is required for Xenopus myogenesis. Dev. Cell 7, 525–534.

Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T., Clevers, H., 2012. Wnt signaling inhibits proteasomal b-catenin degradation within a compositionally intact Axin1 complex. Cell 149, 1245–1256.

Lee, J.M., Dedhar, S., Kalluri, R., Thompson, E.W., 2006. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J. Cell Biol. 172, 973–981.

Logan, C.Y., Nusse, R., 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. 20, 781–810.

Lynch, H.T., de la Chapelle, A., 2003. Hereditary colorectal cancer. N. Engl. J. Med. 348, 919–932.

Luo, W., Ng, W.W., Jin, L.-H., Ye, Z., Han, J., Lin, S.-C., 2003. Axin utilizes distinct regions for competitive MEKK1 and MEKK4 binding and JNK activation. J. Biol. Chem. 278, 37451–37458.

Lilien, J., Balsamo, J., 2005. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. Curr. Opin. Cell Biol. 17, 459–465.

Moon, R.T., Kohn, A.D., De Ferrari, G.V., Kaykas, A., 2004. WNT and beta-catenin signaling: diseases and therapies. Nat. Rev. Genet. 5, 691–701.

Martinez-Arias, A., 2003. Wnts as morphogens? the view from the wing of *Drosophila*. Nat. Rev. Mol. Cell Biol. 4, 321–325.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., Polakis, P., 1995. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. U.S.A. 92, 3046–3050.

Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., Wu, D., 2001. Low-density lipoprotein receptor related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell 7, 801–809.

Mao, B., Niehrs, C., 2003. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. Gene 302, 179–183.

Moolenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O., Clevers, H., 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell 86, 391–399.

Moon, R.T., Bowerman, B., Boutros, M., Perrimon, N., 1998. The promise and perils of Wnt signaling through β-catenin and plakoglobin. J. Cell Biol. 141, 1433–1448.

Miyoshi, Y., Nagase, H., Ando, H., et al., 1992. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum. Mol. Genet. 1, 229–233.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., Kinzler, K.W., 1997. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275, 1787–1790.

Nakaya, Y., Sheng, G., 2013. EMT in developmental morphogenesis. Cancer Lett. 341, 9–15.

Nelson, W.J., Nusse, R., 2004. Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303, 1483–1487.

Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. Nature 287, 795–801.

Nusse, R., Varmus, H.E., 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31, 99–109.

Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., Moon, R., Varmus, H., 1991. A new nomenclature for int-1 and related genes: the Wnt gene family. Cell 64, 231.

Noordermeer, J., Klingensmith, J., Perrimon, N., Nusse, R., 1994. Dishevelled and armadillo act in the wingless signaling pathway in *Drosophila*. Nature 367, 80–83.

Najdi, R., Syed, A., Arce, L., Theisen, H., Ting, J.H., Atcha, F., Nguyen, A.V., Martinez, M., Holcombe, R.F., et al., 2009. A Wnt kinase network alters nuclear localization of TCF-1 in colon cancer. Oncogene 28, 4133–4146.

Narumiya, S., Yasuda, S., 2006. Rho GTPases in animal cell mitosis. Curr. Opin. Cell Biol. 18, 199–205.

Nusse, R., Varmus, H., 2012. Three decades of Wnts: a personal perpective on how a scientific field developed. EMBO J. 31, 2670–2684.

Orford, K., Orford, C.C., Byers, S.W., 1999. Exogenous expression of beta-catenin regulates contact inhibition, anchorage-independent growth, anoikis, and radiation-induced cell cycle arrest. J. Cell Biol. 146, 855–868.

Onorato, T.M., Chakraborty, S., Haldar, D., 2005. Phosphorylation of rat liver mitochondrial glycerol-3-phosphate acyltransferase by casein kinase 2. J. Biol. Chem. 280, 19527–19534.

Panakova, D., Sprong, H., Marois, E., Thiele, C., Eaton, S., 2005. Lipoprotein particles are required for Hedgehog and Wingless signaling. Nature 435, 58–65.

Peifer, M., Wieschaus, E., 1990. The segment polarity gene armadillo encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. Cell 63, 1167–1176.

Peifer, M., McCrea, P.D., Green, K.J., Wieschaus, E., Gumbiner, B.M., 1992. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the *Drosophila* segment polarity gene armadillo form a multigene family withproperties similar. J. Cell Biol. 118, 681–691.

Polakis, P., 2000. Wnt signaling and cancer. Genes Dev. 14, 1837–1851.

Pagliarini, R.A., Xu, T., 2003. A genetic screen in *Drosophila* for metastatic behavior. Science 302, 1227–1231.

Reya, T., Clevers, H., 2005. Wnt signaling in stem cells and cancer. Nature 434, 843–850.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., Nusse, R., 1987. The *Drosophila* homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell 50, 649–657.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R., Bienz, M., 1997. LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. Cell 88, 777–787.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., Polakis, P., 1996. Binding of GSK-3beta to the APC-beta-catenin complex and regulation of complex assembly. Science 272, 1023–1026.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., Weissman, I.L., 2003. A role for Wnt signaling in self-renewal of haematopoietic stem cells. Nature 423, 409–414.

Reilein, A., Nelson, W.J., 2005. APC is a component of an organizing template for cortical microtubule networks. Nat. Cell Biol. 7, 463–473.

Sharma, R.P., Chopra, V.L., 1976. Effect of the Wingless (wg1) mutation on wing and haltere development in *Drosophila melanogaster*. Dev. Biol. 48, 461–465.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., Born, M., Barker, N., Shroyer, N.F., Wetering, M., Clevers, H., 2011. Paneth cells constitute the niche for *Lgr5* stem cells in intestinal crypts. Nature 469, 415–418.

Siegfried, E., Chou, T.B., Perrimon, N., 1992. Wingless signaling acts through zeste-white 3, the *Drosophila* homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. Cell 24, 1167–1179.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., Clarke, A.R., Winton, D.J., 2004. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. 18, 1385–1390.

Su, L.K., Vogelstein, B., Kinzler, K.W., 1993. Association of the APC tumor suppressor protein with catenins. Science 262, 1734–1737.

Sampietro, J., Dahlberg, C.L., Cho, U.S., Hinds, T.R., Kimelman, D., Xu, W., 2006. Crystal structure of a b-catenin/BCL9/Tcf4 complex. Mol. Cell 24, 293–300.

Sekiya, T., Zaret, K.S., 2007. Repression by Groucho/TLE/Grg proteins: genomic site recruitment generates compacted chromatin in vitro and impairs activator binding in vivo. Mol. Cell 28, 291–303.

Shimomura, Y., Agalliu, D., Vonica, A., Luria, V., Wajid, M., Baumer, A., Belli, S., Petukhova, L., Schinzel, A., Brivanlou, A.H., et al., 2010. APCDD1 is a novel Wnt inhibitor mutated in hereditary hypotrichosis simplex. Nature 464, 1043–1047.

Sokol, S., Christian, J.L., Moon, R.T., Melton, D.A., 1991. Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell 67, 741–752.

Sancho, E., Batlle, E., Clevers, H., 2004. Signaling pathways in intestinal development and cancer. Annu. Rev. Cell Dev. Biol. 20, 695–723.

Schuijers, J., Clevers, H., 2012. Adult mammalian stem cells: the role of Wnt, *Lgr5* and R-spondins. EMBO J. 31, 2685–2696.

Strutt, D., Warrington, S.J., 2008. Planar polarity genes in the *Drosophila* wing regulate the localization of the FH3-domain protein Multiple Wing Hairs to control the site of hair production. Development 135, 3103–3111.

Sagner, A., Merkel, M., Aigouy, B., et al., 2012. Establisment of global pattersn of planar polarity during growth of the *Drosophila* wing epithelium. Curr. Biol. 22, 1296–1301.

Thomas, K.R., Musci, T.S., Neumann, P.E., Capecchi, M.R., 1991. Swaying is a mutant allele of the proto-oncogene Wnt-1. Cell 67, 969–976.

Travis, A., Amsterdam, A., Belanger, C., Grosschedl, R., 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor α enhancer function. Genes Dev. 5, 880–894.

Taelman, V.F., Dobrowolski, R., Plouhinec, J.L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., De Robertis, E.M., 2010. Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell 143, 1136–1148.

Turowec, J.P., Duncan, J.S., French, A.C., Gyenis, L., St Denis, N.A., Vilk, G., Litchfield, D.W., 2010. Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. Methods Enzymol. 484, 471–493.

Tsukiyama, T., 2002. The in vivo functions of ATP-dependent chromatin-remodelling factors. Nat. Rev. Mol. Cell Biol. 3, 422–429.

Tolwinski, N.S., Wieschaus, E., 2004. A nuclear escort for beta-catenin. Nat. Cell Biol. 6, 579–580.

Townsley, F.M., Cliffe, A., Bienz, M., 2004. Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat. Cell Biol. 6, 626–633.

Tang, W., Dodge, M., Gundapaneni, D., Michnoff, C., Roth, M., Lum, L., 2008. A genome-wide RNAi screen for Wnt/b-catenin pathway components identifies unexpected roles for TCF transcription factors in cancer. PNAS U.S.A. 105, 9697–9702.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., Nusse, R., 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452.

Willert, K., Nusse, R., 1998. Beta-catenin: a key mediator of Wnt signaling. Curr. Opin. Genet. Dev. 8, 95–102.

Wong, L.L., Adler, P.N., 1993. Tissue polarity genes of *Drosophila* regulate the subcellular location for prehair initiation in pupal wing cells. J. Cell Biol. 123, 209–221.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., Clevers, H., 1997. Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell 88, 789–799.

van de Wetering, M., Oosterwegel, M., Dooijes, D., Clevers, H., 1991. Identification and cloning of TCF-1, a T lymphocyte specific transcription factor containing a sequence-specific HMG box. EMBO J. 10, 123–132.

Waterman, M.L., Fischer, W.H., Jones, K.A., 1991. A thymus-specific member of the HMG protein family regulates the human T cell receptor C alpha enhancer. Genes Dev. 5, 656–669.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., Clevers, H., 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111, 241–250.

Wilkinson, D.G., 2001. Multiple roles of EPH receptors and ephrins in neural development. Nat. Rev. Neurosci. 2, 156–164.

Xing, Y., Clements, W.K., Kimerlamn, D., Xu, W., 2003. Crystal structure of a b-catenin/axin complex suggests a mechanism for the b-catenin destruction complex. Genes Dev. 17, 2753–2764.

CHAPTER

13

Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signaling through Ubiquitinylation

INTRODUCTION

Host defense in mammals is provided by innate and adaptive (or acquired) mechanisms of immunity, both able to discriminate between the self and nonself. In innate immunity, invading microbes are sensed either directly, through the release of cellular components, or indirectly through the deposition of lectins, C-reactive protein, or complement (C3b) on their outer membranes (Kang et al., 2006; Medzhitov et al., 1997). This first line of defense, though not always fully protective, buys time and is required to establish the much more potent processes of adaptive immunity by which invading microbes are sensed by specific immunoglobulins (Ig) and then destroyed. This occurs either directly, by cytolytic antibodies that activate the complement system or by cytotoxic T-cells that recognize virus-infected cells, or indirectly, through stimulation of phagocytosis by granulocytes and macrophages.

In an evolutionary perspective, innate immunity predated adaptive immunity, the dendritic cell constituting the primary cellular link between the two processes. The establishment of adaptive immunity depends on antigen presentation by dendritic cells, macrophages, and B-cells and on the subsequent clonal expansion of antigen-specific T-cells (both CD^{4+} T-helper cells and CD^{8+} cytolytic T-cells) and immunoglobulin-producing B-cells. These processes are dependent on the creation of an inflammatory environment, effectively, the production of inflammatory mediators such as IL1β, IL6, IL12, INFα/β/γ, or TNF and chemotactic factors such as RANTES, MIP1α/β, IL8, or MCP1. These play important roles in the

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recruitment of leukocytes by regulating the expression of adhesion molecules (Chapter 14, "Chemokines and Traffic of White Blood Cells"). They also participate in the maturation of dendritic cells, preparing them to initiate the differentiation of effector T-cells and "memory" cells. Further down the line, the inflammatory mediators also play a role in the maturation of B-cells toward plasma cells, and thus stimulate the production of microbe-specific immunoglobulins.

METCHNIKOV AND EHRLICH

The foundations for the understanding of immunity, both innate and adaptive, were laid by Ilya Metchnikov and Paul Ehrlich, who, in 1908, shared the Nobel prize "in recognition of their work on immunity." We can regard Metchnikov as an early developmental biologist. He detected a population of highly motile cells associated with the larvae of star fish (echinoderms) and postulated that these could somehow be implicated in defense. Putting this idea to the test, he added crusted thorns from a tangerine tree which had served as Christmas decorations for his children, and found the next morning that they were surrounded by these mobile cells. The scene reminded him of white blood cells accumulating at sites of infection and he suggested that the two phenomena represent the mobilization of specialized cells in order to destroy invaders. His friend Professor Claus who suggested the name "phagocytes" (from Greek "phagein," eating) to describe the mobile cells. His first paper on "phagocytosis" appeared in 1884 (Metchnikoff, 1884). Metchnikov immediately realized that the body must have ways to distinguish between self and non-self in order to avoid *horror autoxicus* (autoimmunity). One of *Drosophila*'s antifungal peptides is named after him (see below).

Ehrlich, around 1895, was investigating the protective effect of serum passive immunization against bacterial toxins. He found that it contains "antitoxins" that disable the bacterial products. He was one of the first to recognize the importance of specific molecular interactions or as he stated it: "corpora non agunt nisi fixate," meaning, "substances do not act if they are not bound." The antitoxins, we now recognize as antibodies (immunoglobulins). A similar principle of molecular recognition was also elaborated by John Newport Langley who, in 1906, postulated that "receptive substances" transmit physiological effects (return to Chapter 1, "Prologue: Signal Transduction from a Historical Perspective," Section "The receptive substance").

The work of Ralph Steinman connected the findings of Metchnikov (innate immunity) and Ehrlich (specific antibodies) with the discovery of dendritic cells: they are part of the innate immune system but orchestrate the subsequent adaptive immune response (Steinman and Cohn, 1973).

SENSING THE MICROBIAL UNIVERSE

To initiate an immunological response, the invading microbes must first be recognized. This occurs through a family of "pattern-recognition receptors" (PRRs). These have a limited diversity, are expressed in all cells, and, importantly, function independently of immunological memory. They recognize highly conserved components that are released by invading microbes, collectively named the pathogen-associated molecular patterns (PAMS) (Janeway, 1992). Four different types of receptors are recognized: the cell surface Toll-like receptors (TLRs) (O'Neill et al., 2013) and C-type lectin receptors (CLRs), and the intracellular Nod-like (NLRs) (Chen et al., 2009) and RIG-I-like receptors (RLRs) (Loo and Gale, 2011). We focus on the Toll-like receptors that recognize essential components of microorganisms, such as membrane proteins, lipids, and nucleic acids (Figure 13-1) and Table 13-1 (Rock et al., 1998). More precisely, this chapter deals with the signaling pathway downstream of the Toll-like-4 receptor (TLR4). We note that the intracellular receptors signal through the constitution of an inflammasome, a protein platform that activates caspases-1 or -5 and which subsequently contribute to the process of cleavage-maturation of cytokines such as pro-interleukin-β and -18. These cytokines are secreted without the use of the classic Golgi-produced secretion vesicles (unconventional pathway) (Martinon et al., 2002).

The term PAMS (pathogen-associated molecular patterns) is not really an accurate description because organisms that normally inhabit the body's surfaces (skin and digestive tract) are considered as commensals and not pathogens, but they are also recognized by the PRRs. Of course, they do have to be eliminated in the case that they infiltrate the organism after destruction of the epithelial barrier.

Toll in *Drosophila*: Loss-of-function mutations produce dorsalized *Drosophila* embryos, dominant gain-of-function alleles result in ventralized embryos. The German word Toll means "amazing," "fantastic." Christiane Nüsslein-Volhard wrote to us "When Eric Wieschaus and I first saw the mutant phenotype, we were amazed, because it was so novel and unexpected. I must have yelled 'Toll,' and the name stuck."

Beyond its role in development, Toll plays a key role in the host defense mechanisms of adult flies. While *Drosophila* Toll recognizes the cytokine, spätzle, the mammalian TLRs (TLR1-12) recognize specific microbial components, ranging from single-stranded DNA to lipopolysaccharides (Akira et al., 2006).

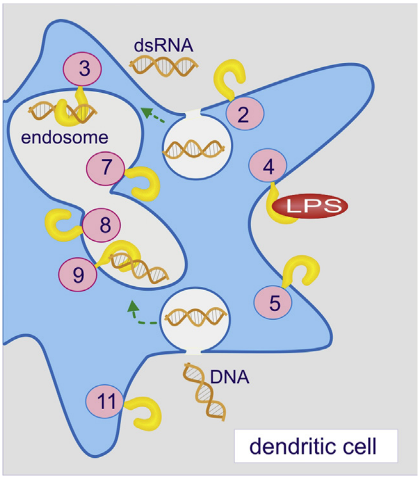

FIGURE 13-1 Subcellular localization of the TLR pattern-recognition receptor family in dendritic cells. TLR 2, 4, 5, and 11 are cell surface receptors that recognize membrane components of invading microorganism. TLR 3, 7, 8, and 9 are present in the endosome compartment where they recognize different forms of nucleic acids taken up by the dendritic cell (see Akira et al., 2006). NB TLR1 and TLR6 are not shown; they are involved in the recognition of diacyl/triacyl lipopeptides and lipoteichoic acid.

TABLE 13-1 Ligands for Toll-like receptors

Ligands	Source organisms	TLR
Viruses		
DNA		9
dsRNA		3
ssRNA		7 and 8
Envelope proteins	RSV, MMTV	4
Hemagglutinin	Measles	2
Bacteria		
Lipopolysaccharides	Gram-negative bacteria	4
Diacyl lipopeptides	Mycoplasma	2 and 6
Triacyl lipopeptides	(Myco)bacteria	1 and 2
Lipoteichoic acid	*Streptococcus bovis*	2 and 6
Peptidoglycan	Gram positive	2
Porins	*Neisseria*	2
Flagellin	Flagellated bacteria	5
CpG-DNA	(Myco)bacteria	9
Fungi		
Zymosan	*Saccharomyces cerevisiae*	2 and 6
Phospholipomannan	*Candida albicans*	2
Mannan	*Candida albicans*	4

TABLE 13-1 Ligands for Toll-like receptors—cont'd

Ligands	Source organisms	TLR
Glucuronoxylomannan	*Cryptococcus neoformans*	2 and 4
Protozoans		
tGPI-mutin	*Trypanosoma*	2
Glycoinositol phospholipids	*Trypanosoma*	4
Hemozoin	*Plasmodium*	9
Profilin-like molecule	*Toxoplasma gondii*	11
Host		
Hsp 60		4
Hsp 90		4
Fibrinogen		4

The Toll receptor in *Drosophila*

The flies possess two strategies of defense against bacterial and fungal invasion: (1) a cellular pathway involving plasmatocytes (the major blood cell type) and (2) a humoral pathway that involves the production of bacteriolytic peptides (such as cecropins, diptericin, drosocin, and defensin), and antifungal peptides (drosomycin and metchnikowin) (Ferrandon et al., 2004).

The events leading to the expression of the drosomycin gene were revealed by comparing signal transduction pathways in mammals and flies that have similar components but are instrumental in completely different processes (Lemaitre et al., 1995; Belvin and Anderson, 1996). Thus, there are striking parallels between the pathway operated by the morphogen "dorsal" in embryonic flies, controlled by the Toll receptor and the mediation of the inflammatory response in mammals controlled by interleukin IL1 (Nusslein-Volhard et al., 1980; Morisato and Anderson, 1995; Mercurio and Manning, 1999). Also, the promoter sequence of the diptericin gene has DNA motifs related to NFKB-binding sites.

The idea that inflammatory responses may be conserved throughout species, and that the dorsal pathway may be replayed by adult flies to control expression of antimicrobial peptides, acted as a spur to investigate the role of the "dorsal/IL-1β" pathway in the regulation of *Drosophila* host defense (Ferrandon et al., 2004). This yielded dividends when it was found that expression of drosomycin occurs through the dorsal pathway (for details see Table 13-2). Ablation of the Toll receptors results in flies that are susceptible to microbial infection, particularly by fungi. The Toll-dorsal pathway is a costimulant for the expression of antimicrobial peptides (Lemaitre et al., 1996).

Spätzle and IL1β are, respectively, the ligands of Toll and IL1R1 (Weber et al., 2003). Although these ligands have no sequence homology,

TABLE 13-2 IL-1β activation of NFκB and the Toll activation of "dorsal"

Process	***Drosophila*, dorsal–ventral patterning and expression of antimicrobial peptides**	**Mammals, inflammatory response**
Ligand	Spätzle	IL1β
Receptor (TIR domain)	Toll	IL1R
Adaptor (TIR/death domain)	Tube	MYD88
Serine/threonine protein kinase (death domain)	Pelle	IRAK4
Inhibitory protein	Cactus	NFKBIA (IkB)
Transcription factor	Dorsal	NFKB
Gene expression	Drosomycin, metchinikowin, cecropin, attacin, defensin	Numerous (see Figure 13-5)

Information from Siebenlist et al. (1994).

spätzle being closer to the mammalian nerve growth factors (NGF), the processing of their inactive precursors is controlled, in part, by immune challenge. During development, spätzle is processed by three extracellular serine proteases ("easter," "gastrulation defective," and "snake"). An alternative proteolytic cascade, activated by an immune challenge, may constitute the activation mechanism in host defense (Levashina et al., 1999). IL1β is processed by an intracellular protease, named interleukin-1β-converting enzyme, ICE (also called caspase-1). In monocytes the activity of this enzyme is controlled by, among other factors, lipopolysaccharides (LPS) (Schumann et al., 1998).

Although the ligands are dissimilar, the receptors, Toll and IL1R share a common intracellular protein–protein interacting TIR domain, through which they bind the adaptors TIRAP, TRIF, TRAM, and MYD88 (in mammalian cells) and MyD88 (in *Drosophila*) (Sims et al., 1989; Heguy et al., 1992) (Figure 13-2). The extracellular domains are completely different. The adaptors Tube and MyD88 both contain a death domain (DD) and are involved in the activation of the serine/threonine kinases pelle or the human homolog IRAK4. The DD is another protein–protein interaction domain also present in the C-terminal region of the TNF receptor (TNFR1) and involved in TNF-mediated signaling for apoptosis (treated in Chapter 14, "Chemokines and Traffic of White Blood Cells"). The DD mediates self-association of these receptors, so directing the signal to downstream events.

Proteins carrying a death domain (DD) are not necessarily involved in the process of apoptosis ("death" signaling). Examples of death domain proteins are IRAK, pelle, tube, ankyrin (cytoskeleton), MyD88, RIP (apoptosis), and TRADD.

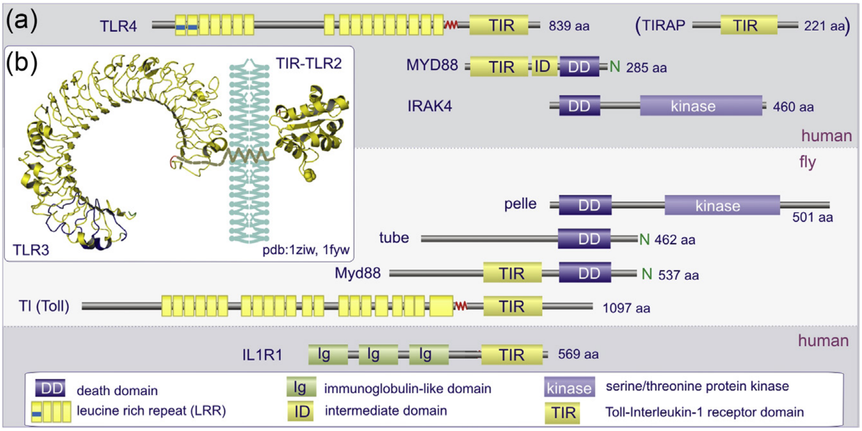

FIGURE 13-2 Comparison of domain architecture of receptors, adaptors, and effectors involved in signaling through the Toll (fly) and Toll-like receptors (human). Toll and TLR4 both have leucine-rich repeats in their extracellular segment (two of which are indicated in blue in panel b) and an intracellular TIR domain. A signaling complex is recruited to the occupied receptor through binding of adaptors containing TIR domains. Those that possess only a TIR domain (such as TIRAP) recruit other TIR-containing adaptors. Adaptors having an additional death domain (DD) recruit the death domain-containing serine/threonine kinase IRAK4 (or pelle in the case of Toll). Note that the IL1R1 also possesses a TIR domain and therefore resembles both Toll and TLR4, but in addition it has three Ig-like domains in the extracellular segment. The N-terminal is normally indicated on the left, with the exception for MYD88, MyD88, and tube.

The TIR domain is the target of an escape strategy of vaccinia virus. It encodes two TIR-domain proteins which block receptor signaling by sequestering MYD88 and TRIF (Heguy et al., 1992).

The downstream targets of these two pathways are Dorsal and NFKB, both of which contain an REL-homology domain (RHD) that binds DNA at the κB sequence motif (first described in the immunoglobulin kappa light chain enhancer region Sen and Baltimore, 1986). Classical NFKB is a transcription factor complex comprising RELA (p65) and a truncated version of NFKB1 (p50). The founding member of this class of transcription factors is v-rel (p58), a viral gene responsible for reticuloendotheliosis in chickens. Mammalian REL-containing proteins include REL (involved in lymphopoiesis), RELA (p65), and RELB but the truncated NFKB1 (p50) and NFKB2 (p52) also qualify because of their REL domain (although not formally) (Figure 13-3).

The *Drosophila* Dorsal gene and the mammalian REL and NFKB1p50/p52 share the characteristic of being sequestered in the cytoplasm by inhibitory proteins; cactus in *Drosophila* and IκB-family members in mammals (ankyrin-repeat proteins). These inhibitors are destroyed by an ubiquitinylation-based process that permits their translocation to the nucleus where they bind to the κB sequence motif.

The Nobel Prize in Physiology or Medicine of 2011 was awarded to Bruce Beutler and Jules Hoffmann "for their discoveries concerning the activation of innate immunity" and to Ralph Steinman "for his discovery of the dendritic cell and its role in adaptive immunity."

LPS: shield and signal

Bacteria that lack an outer membrane have a cell wall composed of a thick layer of peptidoglycan, whereas those bacteria that have an outer membrane have a relatively thin cell wall of lipoproteins and lipoteichoid. This distinction is used as the basis of the Gram test initially devised to discriminate between *Pneumococci* (Gram positive) and *Klebsiella pneumoniae* (Gram negative) (Gram, 1884). It involves staining with crystal violet and iodine to mark the thick layer of peptidoglycan on Gram-positive bacteria (see Table 13-3 for examples of Gram-negative bacteria). *Mycobacteria* form a separate group, being surrounded by a cell wall made up of glycolipids and mycolic acid and with only a thin peptidoglycan layer. They tolerate this because, inhabiting the intracellular environment, they are subject to little osmotic stress.

Penicillin. The cross-linking of the peptidoglycan is targeted by penicillin and results in rapid lysis of microbes.

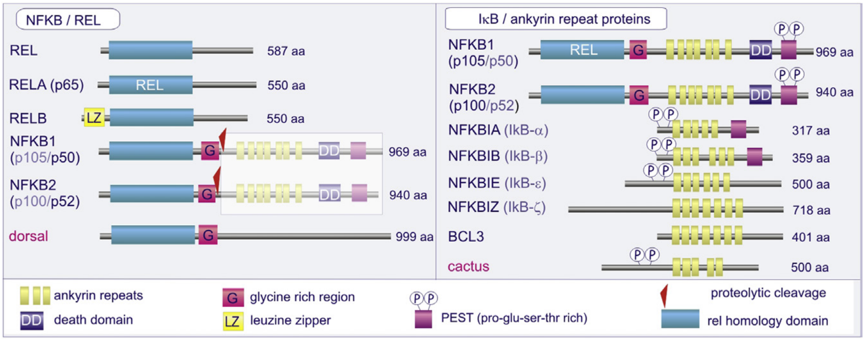

FIGURE 13-3 Domain architecture of the NFκB/Rel and IκB proteins. The Toll and IL-1R pathways control transcription factors of the NFKB/REL family that share an REL-homology domain that determines nuclear localization, DNA binding, and subunit dimerization. Members of this family are held in check by inhibitors, members of the IκB/ankyrin-repeat family, characterized by, as the protein family name indicates, numerous ankyrin repeats. Note that the truncated versions, p50 and p52, are formally ankyrin-repeat proteins, but because of cleavage they also qualify as REL-related proteins. IκB proteins bind NFKB/REL and prevent both recognition of their nuclear localization signal (NLS) and binding to DNA. Partial proteolysis of the ankyrin segment relieves this constraint. Phosphorylation of these proteins signals their destruction and this way allows activation of NFKB. *Figure adapted from Beinke and Ley (2004).*

LPS and mucosa. The form of LPS having secondary acylations (Figure 13-4) allows bacteria to persist in the host mucosa. They are tolerated in this external environment but as soon as they traverse the epithelial barrier, as occurs in trauma, they are effectively eliminated (Guo et al., 1998; Lu et al., 2005; Munford and Varley, 2006).

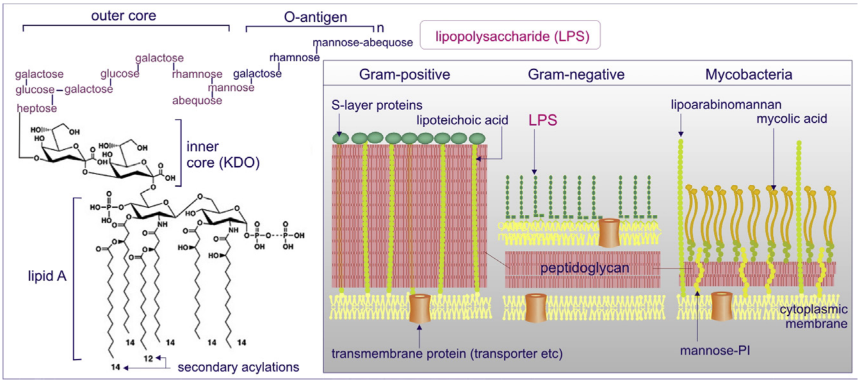

FIGURE 13-4 Composition of the cell wall of bacteria and molecular detail of LPS. Bacteria are protected from the environment by a cell wall. In the case of Gram-positive bacteria, this is composed of a lipid membrane surrounded by a thick layer composed of peptidoglycan, lipoteichoic acid, and a coating of S-layer proteins. Gram-negative bacteria are surrounded by two membranes separated by the peptidoglycan. *Mycobacteria* have a single membrane, a relatively thin peptidoglycan, and a thick outer layer composed of mannose-phosphatidylinositol, lipoarabinomannan, and mycolic acid. *Figure of bacterial cell walls adapted from Akira et al. (2006).*

Virulence of *Yersinia pestis* and acylation of LPS. On entering a host having a body temperature of 37 °C, the plague bacterium *Yersinia pestis* switches production of LPS to a form that lacks secondary acylations. This fails to activate TLR4 and antagonizes the binding of highly acylated LPS. Forced expression of an *Escherichia coli* gene responsible for secondary acylations renders *Y. pestis* completely avirulent. Resistance to disease requires TLR4 and the adaptor protein MyD88. Both innate and adaptive responses are required for immunity against the modified strain. By evading TLR4 activation in this way, the altered LPS may contribute to the virulence of various Gram-negative bacteria (Montminy et al., 2006).

Because the innate immune system lacks any means of instigating receptor diversity, all its targets are highly conserved structural components of microorganisms. One such target is LPS (Galloway and Raetz, 1990). LPS, shed from the outer membrane of Gram-negative bacteria, are detected at picomolar concentrations and serve to alert the host (Figure

TABLE 13-3 Examples of Gram-negative bacteria

Bacteria	Diseases they cause and other remarks
Cyanobacteria	Photosynthesis (3.8 billion years old)
Enterobacter cloacae	Urinary and pulmonary tract infections
Escherichia coli	Commensal in intestine, urinary tract infection, septicemia
Green sulfur and green nonsulfur bacteria	
Helicobacter pylori	Peptic ulcer
Hemophilus influenzae (Pfeiffer bacillus)	Lung infections and meningitis
Klebsiella pneumoniae	Pneumonia, hospital-acquired urinary tract and wound infections
Legionella pneumophila	Legionellosis or Legionnaires'disease
Leptospira icterohaemorrhagiae	Weil's disease
Moraxella catarrhalis	Bronchitis, sinusitis, laryngitis, and otitis media)
Neisseria gonorrhoeae	Sexual transmitted, gonorrhea
Neisseria meningitidis	Meningitis
Proteus mirabilis	Urinary tract infections
Pseudomonas aeruginosa	Pulmonary tract, urinary tract, burns, wounds infections
Salmonella enteritidis	Gastroenteritis
Salmonella typhi	Typhoid fever
Serratia marcescens	Conjunctivitis, keratitis endophthalmitis

13-4). Of the Toll-like receptors, TLR4 has emerged as a specific conduit for the innate immune response to bacterial LPS (Poltorak et al., 1998; Beutler and Poltorak, 2001). We focus on LPS-mediated signaling because it is one of the most potent immunostimulants.

The outer leaflet of the outer membrane of Gram-negative bacteria is made up entirely of lipopolysaccharides. These have a basic structure composed of lipid A, consisting of four to six acyl chains covalently bound to two diamino-dideoxy-D-glucose residues, and a hydrophilic heteropolysaccharide component. The inner component of the polysaccharide, the inner core, is composed of heptose and keto-3-deoxyoctonic acid (KDO). To this, a long chain of polysaccharides is attached, the outer core, of which, the O-antigen, is repeated many times. The long polysaccharide chain is not essential for bacterial survival but the lipid-A moiety is vital. The polysaccharides play a role in pathogenesis and serve as an antigen for the generation of protective antibodies.

LPS and endotoxin. At the start of the twentieth century, long before its chemical composition was realized, Richard Pfeiffer identified LPS as endotoxin. It represents the major fever-evoking component of Gram-negative bacteria and, when applied systemically, it causes shock (Beutler and Poltorak, 2001). We now recognize the lipid A-KDO2 moiety of LPS as being responsible for most of the toxic effects of Gram-negative organisms (Figure 13-4). The immediate pyrogenic effects of endotoxin are due to signaling through TLR4 that elevates the expression of PLA2 and cyclo-oxygenase2 (COX2) and the production of prostaglandin E2 (PGE2) (Steiner et al., 2006). The devastating effects of LPS are not solely due to TLR4-mediated events. Intracellular receptors also exist and these are the proteolytic enzymes caspases-4, -5, and -11. They are activated by LPS, through the formation of an "inflammasome," and cause cell death in monocytes, epithelial cells, and keratinocytes, in a process described as necrosis (not apoptosis) (Shi et al., 2014).

SIGNALING THROUGH THE TLR4 RECEPTOR

The TLR4 differs from other TLRs because it requires the collaboration of other receptors, most notably, CD14 and MD2. LPS, bound to LPS-binding protein (LBP, a trace "acute phase" plasma protein involved in the early stages of septic shock) interacts with CD14 (Schroder and Schumann, 2005; Wright et al., 1990). This presents LPS to MD2, linked to the membrane via TLR4 (Gioannini et al., 2004). Two major signaling pathways are activated downstream of the TLR4 receptor. A signaling complex is formed with a number of adaptors, and the assembly proceeds through the association of proteins via the TIR and DD domains. Many components of the pathway also operate downstream of the TNF, Toll, or TGFβ-receptors (which explains why so many of the acronyms begin with the letter T). One downstream pathway of TLR4 requires the adaptors MYD88 and culminates in the production of pro-inflammatory cytokines (IL1β, IL12, TNFα, and IL6). The MYD88 pathway signals to the transcription factor complexes NFKB, ATF2/JUN (AP1 complex), and IRF5. The other pathway requires the adaptors TICAM2 and TICAM1 (formerly TRAM and TRIF) and culminates in the activation of IRF3, leading to the production of INFβ, IL10, and RANTES. Both pathways converge on the same enhancer region and act in synergy (Figure 13-5).

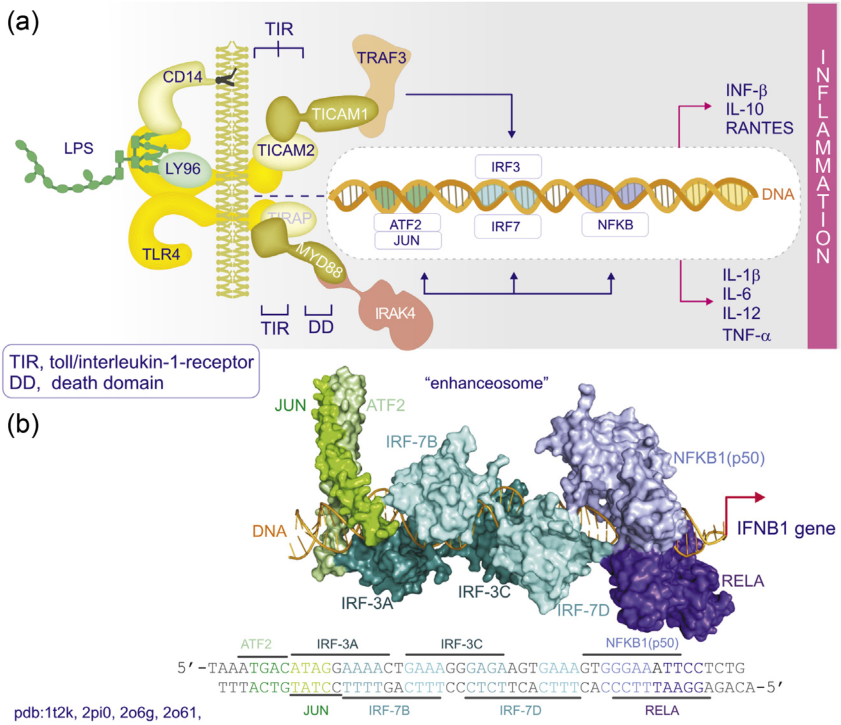

FIGURE 13-5 Signaling from the TLR4 receptor. (a) LPS binds different receptors, first CD14, then MD2, and finally TLR4. Ligand-mediated dimerization (or multimerization) of TLR4 initiates the assembly of receptor signaling complexes through the intermediate of adaptors that harbor the TIR protein-interaction domain. One complex is formed by association of TICAM2 (TRAM) with TICAM1 (TRIF) which, through a death domain (DD), recruits the adaptor/effector TRAF3 (E3-ubiquitin ligase). This causes activation of the transcription factor IRF3 and results in the expression of inflammatory cytokines INFβ, IL10, and RANTES. The other complex is formed by association of the adaptor MYD88 which, through a death domain, recruits the kinase IRAK4. This pathway reaches the transcription factors NFKB, ATF2, JUN, and IRF5 and plays an important role in the expression of IL1β, IL6, IL12, and TNF. (b) Model of the different transcription factors bound to the enhancer region of the interferon-β (IFNB) gene. Note how DNA-binding regions (enhancer elements) overlap between the different proteins and that as a consequence binding to DNA is a function of both protein–DNA and protein–protein contacts. Binding of one transcription factor facilitates that of a second (cooperative binding) and efficient transcription requires the presence of JUN/ATF2, IFR3, IFR7, and NFKB. The structure of the "enhanceosome" is a compilation of a number of PDB entries prepared by Panne et al., and obtained from PDB101 (Panne et al., 2007).

The MYD88 pathway

Oligomerization of the TLR4 receptor provokes the formation of an adaptor complex through the assembly of MYD88, carrying TIR domains. How receptor dimerization creates homophilic TIR–TIR binding sites remains to be elucidated. Individual components have

Discovery of TLR4, the LPS receptor. The earliest indication that LPS might interact with specific receptors came from the discovery of LPS-resistant mice (Heppner and Weiss, 1965). The search for the receptor then commenced in earnest with the realization of two substrains both having defective responses to LPS. Four years of fine mapping of the Lps locus by several research groups culminated in the discovery of LTR4 as the sole candidate (Beutler and Poltorak, 2001). Although LPS interacts directly with TLR4, the interaction also requires one or more co-receptors, among which CD14, a glycosylphosphoinositol-anchored membrane protein expressed on leukocytes (Dauphinee and Karsan, 2006; Wright et al., 1990).

only weak interactions, insufficient to stay together for some time, but multiple interactions reinforce each other and create stable complexes. Thus when the receptor complex attracts two MYD88 proteins, they bind to the receptor (with their TIR domain) and to each other (with their TIR and DD domain). This then attracts new MYD88 proteins and these in turn form a sufficiently large platform to recruit the DD domains of IRAK4 (Figure 13-6). The IRAK4 contributes to the stability of the MYD88 complex and so on. To our understanding this process operates in the auto-assembly of other macromolecular complexes (Lin et al., 2010).

The death domain of MYD88 recruits the kinase IRAK4 which phosphorylates IRAK1 (or IRAK2). Following recruitment on the activated receptor complex, IRAK1 is phosphorylated on Thr-209 resulting in a conformational change of the kinase domain, allowing further autophosphorylations to take place. Thr-387 phosphorylation in the activation loop is required to achieve full enzymatic activity of IRAK1 (Li et al., 2002; Suzuki et al., 2002) (Figure 13-7). The autophosphorylated IRAK1 draws TRAF6 to the receptor complex which now acts as a docking platform on to which another large signaling complex develops. The new docking sites are created, not by phosphorylation as is the case for growth factor receptors, but by a poly-ubiquitin chain, attached in a K63-fashion where the C-terminal glycine (G76) attaches to a Lys-63 of the next ubiquitin (for detail see Figure 13-15). TRAF6 is an E3-ubiquitin ligase that, by association with E2-conjugating enzymes (a complex of UBE2N/UBE2V2), causes the ubiquitinylation of itself (on Lys-125) and of IRAK1 (on Lys-134 or -180, in the region between DD- and protein kinase domain) (Deng et al., 2000; Eddins et al., 2006; Yin et al., 2009). Importantly, TRAF6 and IRAK1, decorated with their poly-ubiquitin chains, bring together two kinase complexes that act in series to ensure phosphorylation of the inhibitor of NFKB (NFKBIA) and activation of the JNK and p38 protein kinase pathways.

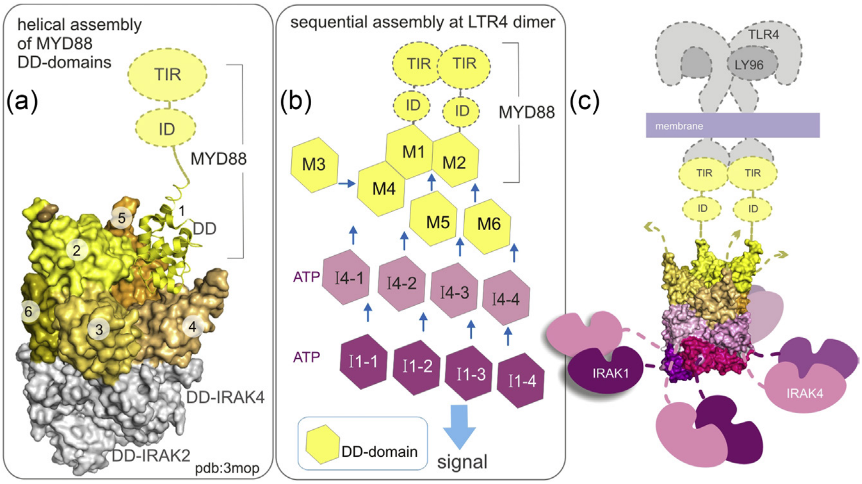

FIGURE 13-6 Assembling receptor signaling complexes through multiple reenforcing interactions. (a) Molecular structure of a DD-domain assembly. In different tints of yellow the DD domains of MYD88, numbered from 1 to 6. Note that they are arranged in a helical fashion, starting on top with 1 and winding down (anticlockwise) to 6. (b) Schematic representation of the sequential assembly process, where the TLR4 receptor dimer brings together the DD domains of MYD88, first M1 and M2 which then recruits M3 to M6. These in turn attract IRAK4 (I4-1) leading the stabilization of the MYD88 complex. Finally IRAK4 recruits the DD domain of IRAK1 (I1-1). (c) "Artist impression" of the TLR4 signaling complex. The assembly of multiple protein kinases causes the activation of IRAK1. From here the signal now continues into the cell. *Image adapted from Lin et al. (2010).*

The significance of IRAK4 is clearly illustrated in patients that either fail to express it, or have a mutation in its kinase domain which renders the enzyme inactive. Due to ineffective innate immunity, these people are particularly susceptible to infection with *Streptococcus pneumoniae*, suffering recurrent pyrogenic infections and bacteremia (Picard et al., 2003).

From TRAF6 to activation of NFKB

The first complex recruited by the K63-ubiquitin chain attached to either TRAF6 or IRAK1 is composed of the serine/threonine kinase MAP3K7 (also known as TAK1) with its associated adaptor (TAB2 or TAB3) and its regulatory subunit TAB1 (both MAP3K7/TAB1/TAB2 and MAP3K7/TAB1/TAB3) complexes have been detected (Takaesu et al., 2000; Wang et al., 2001). It is the TAB2 that binds the K63-ubiquitin chain through a CUE zinc-finger domain (Kanayama et al., 2004) (Figure 13-8 illustrates the domain architecture of these proteins). This activates

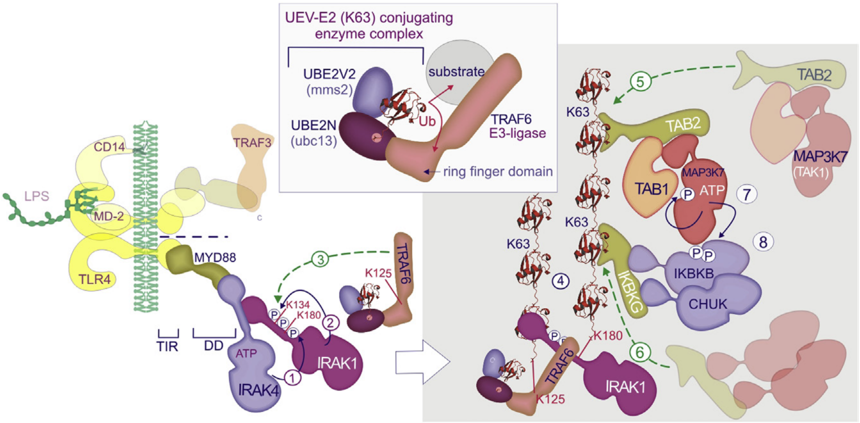

FIGURE 13-7 TRAF6-mediated signaling complex formation. Recruitment of IRAK4 brings IRAK1 into the signaling complex. IRAK4 phosphorylates IRAK1 (threonine-209) (1) and this brings about a conformational change that allows IRAK4 to phosphorylate itself on numerous residues in particular in the region in between the death domain and the kinase domain (pro-ser-thr-rich sequence) (2). This in turn causes the recruitment and activation of an ubiquitin-transfer complex (3), comprising TRAF6 (E3-ligase) and UBE2N (formerly Ubc13) and UB2V2 (formerly mms2). The latter two constitute the EUV-E2-conjugating enzyme complex (see insert). Both IRAK4 (on K134 or K180) and TRAF6 (on K125) are substrates. The UBE2N/UB2V2 enzyme connects the ubiquitins via their C-terminal glycine (G76) and lysine in position 63 (K63) (4). The K63-ubiquitin chain acts as a docking site for two kinase complexes. One of these comprises TAB2 coupled to the serine/threonine kinase TAK1 (gene name MAP3K7) together with its regulatory subunit TAB1 (5). The other comprises IKBKG (formerly NEMO) attached to IκB-kinases-α and -β (CHUK and IKBKB). Recruitment of TAB2/TAB1/TAK1 results in autophosphorylation of the kinase (7) and subsequent phosphorylation (on serine-177 and -181) and activation of IKBKB (8).

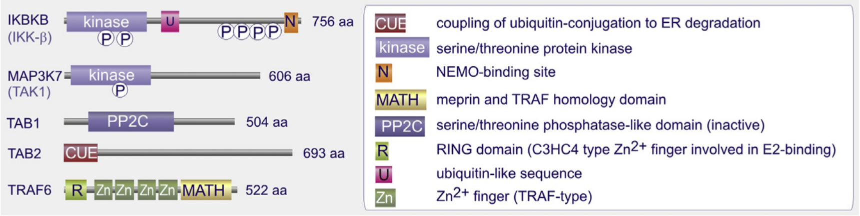
IKBKB (IKK-β)
kinase
U
N
P P
P P P P
756 aa
MAP3K7 (TAK1)
kinase
P
606 aa
TAB1
PP2C
504 aa
TAB2
CUE
693 aa
TRAF6
R Zn Zn Zn Zn MATH
522 aa
CUE coupling of ubiquitin-conjugation to ER degradation
kinase serine/threonine protein kinase
N NEMO-binding site
MATH meprin and TRAF homology domain
PP2C serine/threonine phosphatase-like domain (inactive)
R RING domain (C3HC4 type Zn2+ finger involved in E2-binding)
U ubiquitin-like sequence
Zn Zn2+ finger (TRAF-type)

FIGURE 13-8 Domain architecture of proteins involved the TRAF6-mediated activation of the IKK complex. The phosphorylation sites in the kinase domain of IKBKB and MAP3K7 (TAK1) are situated in the activation segment. The MAP3K7 regulatory subunit TAB1 has a phosphatase fold similar to PP2C but is inactive. TAB2 binds K63-linked ubiquitin through its CUE domain. TRAF6 possesses an N-terminal RING domain with which, together with the first two Zn-motifs, it binds to the E2-conjugation complex (UBE2N and UBE2V2) (not shown).

MAP3K7 through autophosphorylation (Kishimoto et al., 2000). The second complex is composed of the serine/threonine kinases CHUK (IKKα) and IKBKB (IKKβ), linked to the ubiquitin-binding protein IKBKG (IKKγ also known as NEMO) that acts as the sensor for the K63-ubiquitin chain (IKK signifies IκB kinase, a complex of three proteins) (DiDonato et al., 1997; Wu et al., 2006). Upon binding to TRAF6, MAP3K7 phosphorylates and activates IKBKB (on Ser-177 and -181) which then phosphorylates the inhibitor of NFKB (NFKBIA, also known as IκBα) in the "destruction box" (Figure 13-9) (Israel, 2010). The phosphorylated NFKBIA is detected by the receptor-component BTRC of an E3-ligase complex and this results in polyubiquitinylation (of the K48-type). The mechanism of marking proteins for destruction and the component of the E3-ligase complex (SCF$^{\text{B-TRC}}$) has been discussed in Chapter 11, "Signal Transduction to and from Adhesion Molecules," see Figure 11-19, and in Chapter 12, "WNT Signaling and the Regulation of Adhesion and Differentiation," see Figure 12-8.

On destruction of NKKBIA, the NFKB-dimer—comprising RELA and NFKB1p50—is recognized by the α- and β-importins (KPNA1 and KPNB1) at the nuclear pore and enters the nucleus (Chen, 2005). This pathway of activation of NFKB, through destruction of its inhibitor, is referred to as

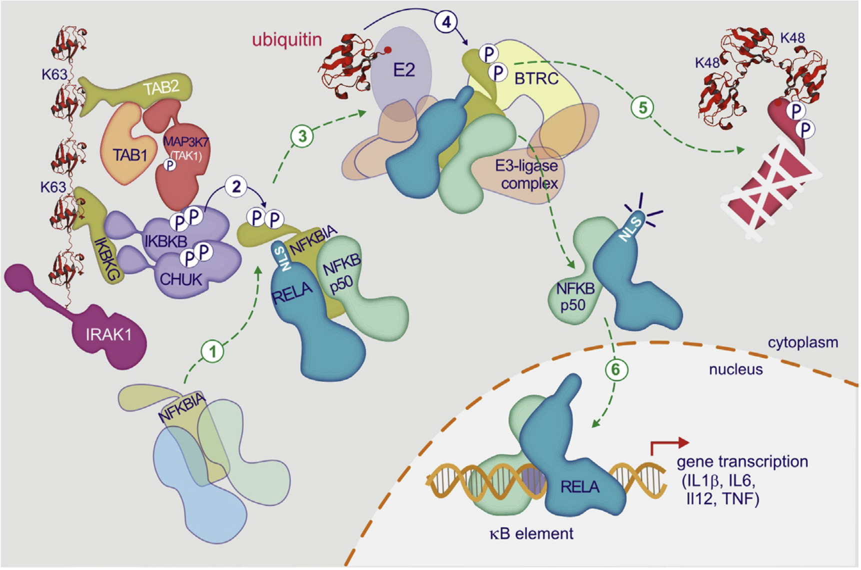

FIGURE 13-9 IKBKB-mediated activation of the RELA/NFKBp50 complex. Phosphorylated/activated IKBKB phosphorylates IκB on two serine residues in its N-terminal domain allowing its recognition by the SCF$^{\text{BTRC}}$ ubiquitin–ligase complex. Following ubiquitinylation (K48-type), NFKBIA (IκBα) is marked for destruction by the proteasome, liberating RELA (p65) and NFKB1p50 and exposing their nuclear localization signal (NLS). They enter the nucleus, there to bind DNA at the κB element, driving expression of inflammatory cytokines.

the "canonical NFKB pathway." We have given here the example NFKBIA but the other inhibitors, NFKBIB, -C, -E, and -Z, can also bind NFKB thereby blocking the nuclear localization signal and thus transcriptional activity (Sen and Baltimore, 1986). Return to Figure 13-3 for the list of ankyrin-repeat proteins involved in the control of NFKB signaling.

Alternative activation pathways for NFKB

These pathways involve unprocessed (precursor) members of the ankyrin-repeat family, NFKB1 and NFKB2. Remember, besides their ankyrin-repeats, these proteins also carry an REL domain (return to the right column of Figure 13-3). They bind to other members of the REL family and, having an intrinsic inhibitory region that masks the nuclear localization signal, they render the complexes inactive with respect to their transcriptional function (Beinke and Ley, 2004). The NFKB1p105 pathway involves the unprocessed NFKB1p105 which preferentially binds to a homodimer of processed NFKB1p50. The complex is inactive, and ubiquitinylation of p105 followed by total destruction is required for liberation of the processed NFKB dimer.

In the non-canonical (or alternative) pathway, NFKB2 is bound to RELB and is phosphorylated in its destruction box by dimer of CHUK (IKKα). Poly-ubiquitinylation is then followed by only partial proteolysis through the proteasome. This processed form of NFKB2p52 translocates, in association with RELB, to the nucleus. This pathway operates mainly in B cells in response to a subset of TNF receptors, including BAFF, LTα, LTβ, and CD40 (Xiao et al., 2001; Sun, 2011).

From TRAF to activation of MAPK8 (JNK1) and MAPK14 (p38α)

In parallel with the activation of NFKB, two other pathways contribute to the transcriptional regulation of inflammatory mediators (Ninomiya-Tsuji et al., 1999). MAP3K7 phosphorylates the activation segments of MAP2K3 (MEK3) and MAP2K4 (MEK4) which then phosphorylate and activate MAPK14 (p38α) and MAP8 (JNK1) (respectively) (Figure 13-10). These members of the big MAP-kinase family are involved in the responses to cellular stress and inflammation (return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Figure 10-27).

MAPK8 (JNK1 or JUN N-terminal kinase) enters the nucleus to phosphorylate the transcription factors ATF2 (a member of the ATF/CREB family of transcription factors) (Sano et al., 1999) and JUN (member of the AP-1 family of transcription factors). For more information return to Chapter 7, Figure 7-9 and Chapter 9, Figure 9-10. Numerous genes carry response elements in their promoter regions that bind these transcription factors. MAPK14 (p38α) remains in the cytosol and acts to enhance inflammatory cytokine production through phosphorylation and activation of MAPKAP2 (MK2) (a member of the MAP-kinase-activated protein

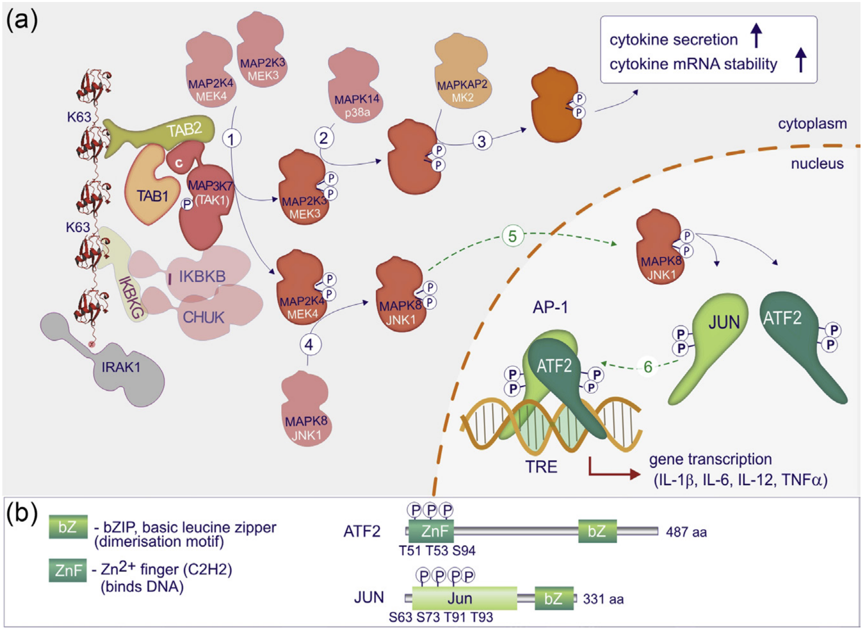

FIGURE 13-10 MAP3K7-mediated activation of MAPK14 (p38α) and MAPK8 (JNK1). (a) MAP3K7 phosphorylates MAP2K3 (MEK3) and MAP2K4 (MEK4) (1). These in turn phosphorylate and activate, respectively, MAPK14 (p38α) (2) and MAPK8 (JNK1) (4). P38α phosphorylates and activates MAPKAP2 (MK2) (3) which enhanced expression of inflammatory cytokines through stabilization of their mRNAs. It also promotes their secretion. MAPK14 enters the nucleus and phosphorylates the transcription factors ATF2 and JUN (5). These form heterodimers (AP-1 complex) that bind the TRE (TPA response element) (6) and drive expression of numerous inflammatory cytokines. Note that the same enhancer region also contains enhancer elements that bind NFKB and IRF (return to Figure 13-5). (b) Domain architecture of ATF2 and JUN. The N-terminal DNA-binding regions of the proteins are phosphorylated by MAPK14. They dimerize through a basic leucine zipper (bZIP) in the C-terminal segment.

kinases) that acts to enhance the expression and secretion of inflammatory cytokines through stabilization of the relevant mRNAs.

From TRAF6 to activation of IRF5

The TRAF6 signaling complex also causes dimerization of the interferon-regulatory factor IRF5 which is then transferred into the nucleus. Mice lacking IRF5 are resistant to the lethal effects of LPS (Takaoka et al., 2005). Further down we will elaborate on IRFs.

The TICAM2, TICAM1, TRAF3 pathway

Here we concentrate on the role of TRAF3 (TNF-receptor-associated factor), an E3-ligase associated with UBE2N and UVE2V2 (formerly known as ubc13 and mms2, respectively) (Zeng et al., 2009) in the recruitment of another kinase complex to the TLR4 receptor. Lacking the involvement of MYD88, it is also known as the MYD88-independent pathway (Figure 13-12). It is suggested that the MYD88-independent pathway described below is initiated exclusively on endosomes, after uptake of the LPS-bound receptors (see Hacker et al., 2011). The TLR4 dimer recruits TIR domain-containing proteins, TICAM2 (formerly known as TRAM) and TICAM1 (TRIF) and these probably assemble in an oligomer complex as shown for MYD88 (Figure 13-6). TICAM1 then recruits TRAF3, an E3-ligase that associates with an E2-conjugation complex composed of EBE2N/UBE2V2 (similar as described for TRAF6). We note that despite the fact that TRAF3 recruits a genuine E2-conjugation complex it is not an approved E3-ligase. It qualifies as an adaptor protein. E2-conjugating enzyme EBE2N/UBE2V2 makes K63-linked ubiquitin chains which are added to TRAF3 on lysines-107 and/or -156. The ensuing K63-ubiquitin chain attracts TANK (TRAF-associated NFKB activator), an adaptor protein which plays the same role as earlier described for IKBKG (NEMO). It is associated with a protein kinase, either IKBKE (IKK-ε) or TBK1 (TANK-binding kinase-1) (Fitzgerald et al., 2003). Multiple TANK-kinase complexes are associated with the long K63-ubiquitin chain and so multiple kinases are brought together. Incidentally, TICAM1 and -2 also bind the transcription factors IRF3 which may play a role in getting the substrates near the relevant protein kinases (Figures 13-11 and 13-12).

Both IKBKE and TBK1 have a kinase fold that resembles that of other members of the IKK family of kinases, namely IKBKA and -B (IKKα and -β) (Chariot et al., 2002). Recruitment of TBK1 and the ensuing activation process merits further explanation. The kinases form dimers that are stabilized by an extensive network of interactions among the kinase domain, the ubiquitin-like domain (ULD), and the triple helical coiled-coil scaffold/dimerization domain (SDD). They possess an activation segment that holds the kinase in a catalytically incompetent configuration (for instance, the highly conserved glutamate (E55) in the αC-helix points in the wrong direction). Recruitment in the receptor signaling complex causes two dimers (each attached to TANK) to face each other resulting in a swap of activation segments; one enters the catalytic cleft of the other and acts as a substrate, and vice versa. Both kinases being relieved of their constraint phosphorylate the Ser-172 of their neighbor (phosphorylation *in trans*) and this brings about a conformational change that causes the opposing kinases to detach from each other. The phosphorylated dimers constitute the active protein kinase. Two such events have to occur in order to obtain two active protein kinases in the dimer complex (Figure 13-11) (Larabi et al., 2013; Ma et al., 2012). We note that TBK1 undergoes K63-linked ubiquitinylation at K30 (kinase domain) and K401 (SDD) and at least one is required for kinase activation, so the story is still unfinished (Tu et al., 2013).

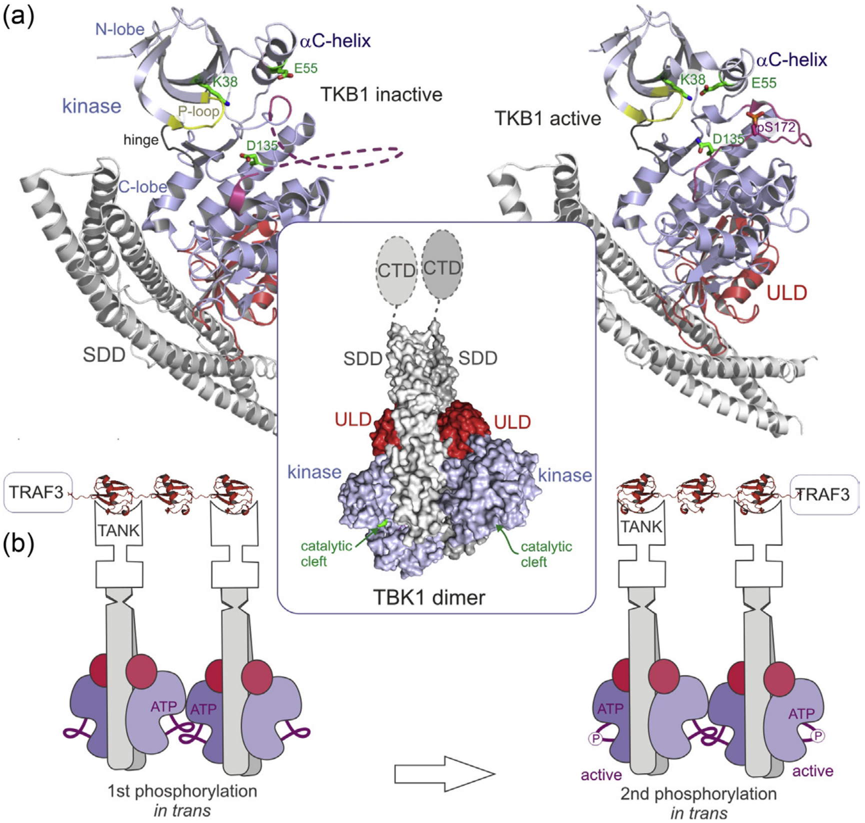

FIGURE 13-11 Structure of TBK1 and its mode of activation. (a) The TBK1 kinase occurs in a catalytically incompetent state as witnessed by the outward position of the αC-helix that harbors a highly conserved glutamate (E55) involved in the coordination of ATP. This changes upon phosphorylation of serine-172 in the activation segment leading to an activated protein kinase. The protein comprises four subregions, a kinase domain, an ubiquitin-like domain (ULD), a scaffold/dimerization domain (SDD), and a C-terminal domain (protein-interaction domain). They form a homodimer through multiple interactions between the SDD, ULD, and kinase domains (inset). (b) Proposed model for the activation of TBK1. The C-terminal domain binds TANK and connects the protein kinase dimer with the TRAF3-associated K63-ubiquitin chain. When two dimers face each other they exchange their activation segment and this renders the protein kinases competent. They phosphorylate each other (*in trans*) on Ser-172. Upon dissociation they now remain fully active. The process can be repeated for phosphorylation of the second kinase in the dimer. Pdb coordinates of the dimeric TKB1 were kindly provided by Dr Daniel Panne, European Molecular Biology Laboratory, Grenoble, France. *Image adapted from Larabi et al. (2013) and Ma et al. (2012).*

As a consequence of TBK1 activation, IRF3 gets phosphorylated on multiple residues, in the N-terminal sequence, and, of these, phosphorylation of S386 is considered essential for transcription factor activation. This means that the proteins obtain the quality to form dimers, to hide the nuclear-export signal, and to bind DNA (Shu et al., 2013). The phosphorylated

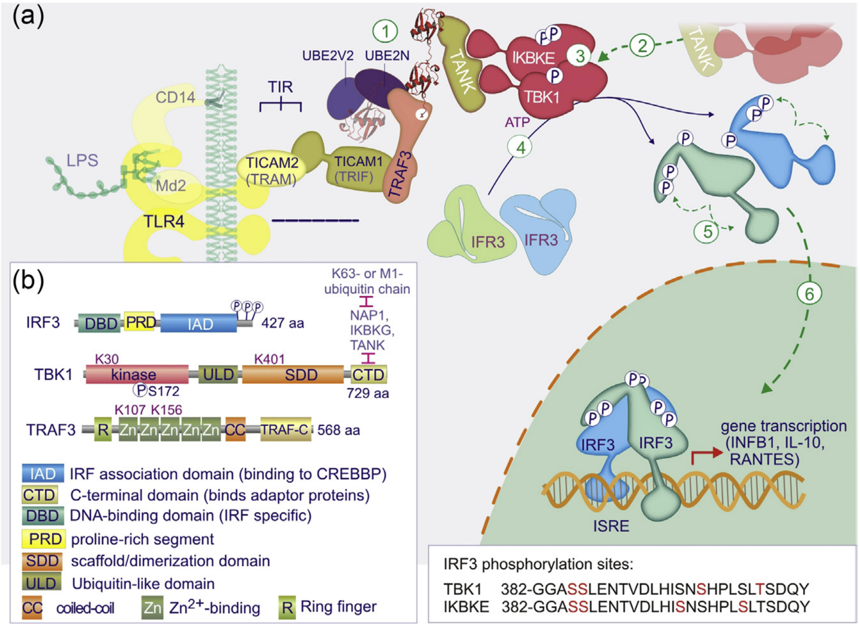

FIGURE 13-12 TICAM-mediated receptor signal complex formation and the activation of IRF3. (a) TLR4 recruits TICAM2 (TRAM) and TICAM1 (TRIF) through their TIR domains. TICAM1 recruits an ubiquitinylation complex composed of TRAF3 (likely to be the E3-ligase), UB2N, and UB2V2 (E2-enzyume) which in turn, binds the adaptor TANK coupled to IKBKE (IKK-ε) and TBK1 (1). The formed complex causes activation of TBK1 through a transphosphorylation process described in Figure 13-11. It is unclear to us what happens to IKBKE. TBK1 phosphorylates the N-terminal region of IRF3, which unfolds both the DNA and the transactivation (IAD) domains of the protein. IRF3 forms a homodimer that enters the nucleus, binding to the ISRE (interferon stimulated response element). (b) Domain architecture of components of the IFR3 activation pathway. Activation phosphorylation sites of IRF are at the C-terminal in and just outside the IRF association domain (IAD) that binds the transcription factor CBP. TBK1 is different from IKK-ε with respect to its activation segment, containing only a single serine phosphorylation site. TRAF3, like TRAF6, is an E3-ligase, with a RING domain (R) that binds the E2-conjugating enzyme. As illustrated here, it merely acts as an adaptor. Its role as ubiquitylating enzyme in the TLR4 pathway remains unclear.

homodimers (or heterodimers IRF3/IRF7) translocate to the nucleus to bind DNA at the interferon-stimulated response element (ISRE) (Figure 13-12).

The IRF3 homodimer or IRF3/7 heterodimer binds to CREBBP which, as we have discussed in Chapter 7, "Bringing the Signal into the Nucleus," is an essential co-activator for gene transcription. Transcriptional activity of IFR3 requires the cooperation of NFKB (return to Figure 13-5). All this leads to enhanced expression of the interferon-β gene (IFNB1) but other genes are also expressed through this pathway such as IL10 and the chemokine RANTES. Absence of IFR3 prevents the production of INFB1 and IL10 without reducing the expression of the pro-inflammatory cytokines TNFα, IL1β, or IL6. Indeed, their production is actually enhanced, possibly due to the failure of negative control normally exerted by IL10 (Hacker et al., 2006).

In addition to their role in innate immunity, TBK1 and IKBKE (IKKε) contribute directly to cell transformation (Shen and Hahn, 2011). IKBKE is a breast cancer oncogene that is amplified in 30% of breast cancers. In these cancers, IKBKE-mediated activation of NFKB signaling is required for transformation, at least in part through phosphorylation and inactivation of the tumor suppressor CYLD and TRAF2. In cancers involving activation of KRAS mutations, RALB-mediated activation of TBK1 promotes cell survival.

THE IRF FAMILY OF TRANSCRIPTION FACTORS

The interferon regulatory factors (IRF's), first identified in the promoter region of the human interferon-β gene (IRFB1) (Miyamoto et al., 1988), operate in both innate and adaptive immunity. They recognize a consensus DNA sequence known as the interferon-stimulated response element (ISRE) and are functionally active as homo- or heterodimers. Actually, their application is widespread, with many genes containing the ISRE consensus sequence and they are activated by several receptors, including TLR4 (Honda and Taniguchi, 2006). The mammalian IRF family comprises nine members (IRF1-9). Except for IRF1 and IRF2, their C-termini (IAD domain) resemble the SMAD family of transcription factors suggesting interactions with other regulatory transcription factors or with the general machinery of gene transcription (Lohoff and Mak, 2005). Different IRFs play different roles as exemplified by the phenotypes of knockout mice. Loss of IRF7 renders mice vulnerable to viral infection, correlating with a decrease in serum interferon levels (Honda et al., 2005). Loss of IRF5 has little effect on INFA1 production but the animals are resistant to the lethal effect of systemically applied non-methylated DNA or LPS and this correlates with a reduction of serum IL6, TNFα, and IL12 (Takaoka et al., 2005).

NEGATIVE FEEDBACK CONTROL OF THE TLR4 PATHWAY

We present five ways by which the TLR4 pathway is held in check. These involve either an activated protein kinase that feeds back on upstream components or they involve gene products that are induced by the TLR4 pathway (Figure 13-13):

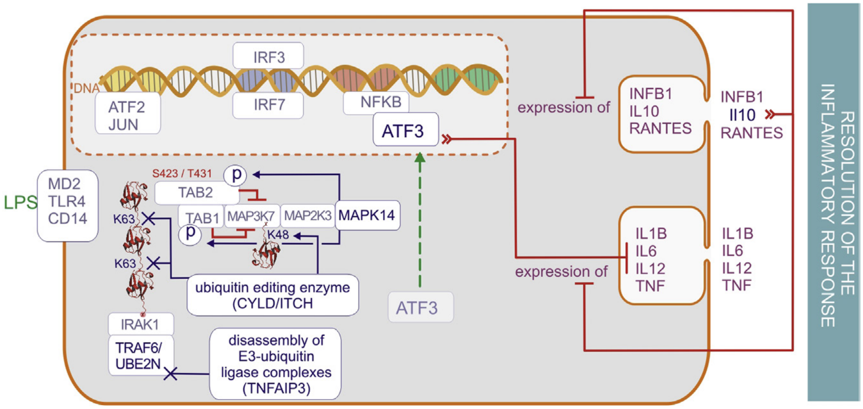

FIGURE 13-13 Negative feedback of the TLR4 signal transduction pathways: resolution of the inflammatory response. Among the genes regulated by the different transcription factors are ubiquitin editing proteins (having both protease and ligase functions) that cleave the K63-ubiquitin isopeptide bonds (on TRAF3, TRAF6, and IRAK1) and simultaneously add K48-chains to MAP3K7 (function of CYLD bound to ITCH). Others remove the K63-chain of TRAF6 and add K48-chain to its E2-ligase partner UBE2N (function of TNFAIP3 bound to RNF11/ITCH). Their action causes disassembly of the TRAF6-mediated signaling complexes. Activated MAPK14 (p38α) phosphorylates the ubiquitin-binding protein TAB2 as well as the regulatory subunit TAB1. Both phosphorylations cause inactivation of MAP3K7 (TAK1). ATF3 is also one of the targets of the TLR4 signal pathways, acting to suppress transcription of IL6 and IL12 when bound to NFKB and ATF2/JUN. Finally, IL10 has a general inhibitory effect on expression of pro-inflammatory cytokines. Collectively, these negative feedback pathways act to resolve the inflammatory response.

1. The transcription factor ATF3 participates in the same transcription complex as NFKB and ATF2, acting as a negative regulator of IL6 and IL12. It does this by altering the chromatin structure, thereby restricting access to the other transcription factors.
2. Inhibition of MAP3K7 (TAK1) by phosphorylation of its regulators TAB1 and TAB2 by MAPK14 (p38α) (Cheung et al., 2003).
3. Expression of de-ubiquitinating enzymes which break the K63-isopeptide link in the ubiquitin chains attached to TRAF3, TRAF6, and IRAK1. An example is CYLD. When bound to ITCH, an E3-ubiquitin ligase, it also causes K48-ubiquitinylation of MAP3K7 (TAK1) and causes its destruction (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Wertz, 2011).
4. Expression of proteins that disassemble ubiquitin ligase complexes, example of TNFAIP3 (also known as A20). In cooperation with TAX1BP1 it promotes disassembly of E2–E3 ubiquitin protein ligase complexes in the Toll-like receptor, TNF, and IL1B pathways, acting on the E3-ligases BIRC2, TRAF2, and TRAF6. With respect to TRAF6 it interferes with the E2 ubiquitin-conjugating enzymes UBE2N and UBE2D3. Moreover, the

binome TNFAIP3/TAX1BP causes K48-uniquitination of UBE2N which causes its destruction by the proteasome. Increased activity of TNFAIP3 protects mice from endotoxic shock, demonstrating the critical function of inhibitors of K63-ubiquitinylation in the restriction of TLR signals in immune cells (Boone et al., 2004).

5. Expression of the cytokine IL10 which terminates the inflammatory response by deactivating macrophages and effector T-cells and silencing the synthesis of TNF, IL6, IL1β, and an array of other chemokines (Moore et al., 2001).

SOME CONSEQUENCES OF TLR4-INDUCED GENE TRANSCRIPTION

Below we list some of the mediators generated following provocation by foreign organisms and activation of PRRs. Although many cells contribute to mediator production and therefore contribute to the establishment of the adaptive immune response, the dendritic cell must be regarded as the primary cellular bridge linking innate and adaptive immunity. As the major antigen presenting cell, it signals the T-effector cells and thereby triggers the production of antigen-specific immunoglobulins.

- INFB1 is necessary for the maturation of antigen-presenting dendritic cells: increased expression of MHCII and co-stimulatory proteins, including CD40, CD80, and CD86 (Chapter 15, “Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases”).
- INFB1 and IL12 are necessary for formation of Th1 cells, important in antigen-specific activation of B-cells, leading to the production of specific antibodies.
- IL6 stimulates the maturation of B-cells toward high level immunoglobulin-producing plasma cells.
- TNF and IL1B are essential for the increased expression of adhesion molecules on endothelial cells, leading to enhanced extravasation of leukocytes and dendritic cells at sites of infection (see Chapter 14, “Chemokines and Traffic of White Blood Cells”).
- Several chemokines (RANTES, MIP1B, IL8, CCL5, CXCL9, etc.) cause activation of integrins on leukocytes, including dendritic cells. This leads to their enhanced extravasation and tissue infiltration in order to combat the infecting agent.

In Chapter 14, “Chemokines and Traffic of White Blood Cells,” we show how the TLR4 response leads to recruitment of leukocytes at sites of infection. In Chapter 15, “Activating the Adaptive Immune System“ we explain how the innate immune response establishes an adaptive immune response, involving the antigen-presenting molecule MHCII and signaling downstream of the T-cell receptor (TCR).

ESSAY: UBIQUITINYLATION AND SUMOYLATION

Ubiquitinylation

Ubiquitinylation is the process by which the peptide ubiquitin (76 amino acids) is coupled to the epsilon-amino groups of lysines in the target protein (Figure 13-14). There are four genes in the human genome that produce ubiquitin; UBB, UBC, UBA52, and RPS27A. These either give rise to a poly-ubiquitin that is subsequently cleaved in three ubiquitins (case of UBB and UBC) or they code for a chimer, ubiquitin, and a small 40S-ribosomal protein, which is also cleaved (case of UBA52 and RPS27A) (Kimura and Tanaka, 2010). They came to light in studies concerning the role of ATP-driven processes in the control of protein degradation. It is self-evident that it must specify its targets with great accuracy: were it not, protein degradation would be uncontrolled. The reaction is reversible (Nijman et al., 2005).

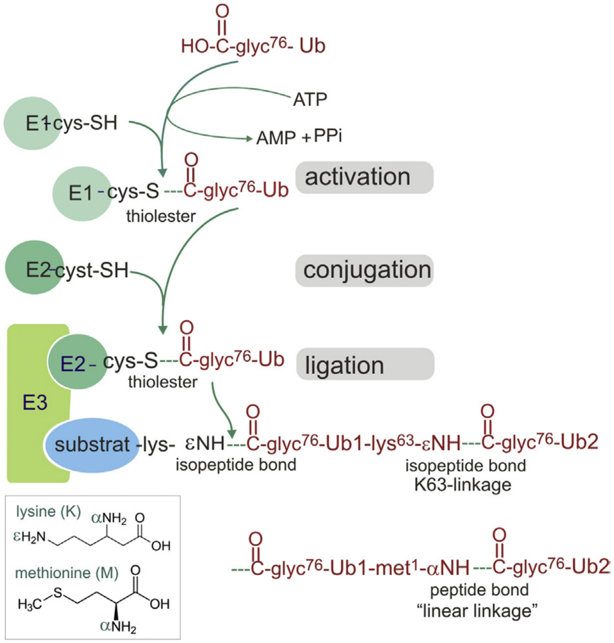

FIGURE 13-14 The ubiquitinylation reaction. The ubiquitinylation reaction occurs in three steps. Following adenylation ("activation"), ubiquitin is bound to E1-cysteine through a thioester bond. This allows transfer of the ubiquitin to E2-cysteine (residue-86 in this example). With substrate bound to E3, the ubiquitin hops onto a lysine ε amino group to create a stable isopeptide bond. This reaction can be repeated by addition of further ubiquitins which may be attached to each other through one of the seven available εNH2 lysine groups in ubiquitin (see also Figure 13-15) or through the αNH2-group of the initiator methionine (M1). In the latter case this gives rise to a so-called linear chain. Poly-ubiquitin chains, rather than just one ubiquitin, are required for protein destruction or assembly of signaling complexes.

As has become evident in this chapter, the attachment of one or more ubiquitins does however much more than just marking proteins for destruction by the proteasome complex. It also serves as a signal for endocytosis of cell surface receptors and subsequent selection for lysosomal destruction. It is involved in the regulation of protein kinases, it regulates gene transcription, DNA repair, and it acts as a protein–protein interaction motif involved in the recruitment of signaling complexes (Liu, 2004).

Ubiquitin conjugation is catalyzed in three steps. Similar to adenylation, ubiquitin is first "activated," bound to E1-cysteine through a glycine-sulfydryl thioester link. It is then transiently coupled to a cysteine on the E2-conjugating enzyme. The substrate (target protein) and E2-ubiquitin are brought in close proximity through a third component, E3-ligase, which determines the specificity of the conjugation reaction (Figure 13-14). Ubiquitin detaches from E2 to form an isopeptide bond linking the α-carboxyl group of its terminal glycine with an ε-amino group of a lysine residue in the substrate.

Different conjugations

Repeated conjugation results in the formation of poly-ubiquitin chains. Ubiquitin itself possesses seven lysines, all of which can make the link to the N-terminal glycine, through isopeptide bonds, but two of these, Lys48 and Lys63, predominate (48K- or 63K-type). Recognition of target proteins by the proteasome complex requires four or more linked 48K-type ubiquitins and perhaps all other possible conjugations, with the exception of K63 and M1 (see below) (Figure 13-15). The 63K and M1 poly-ubiquitin chains are involved in signaling complex assembly, serving more readily as a docking site for other ubiquitin-binding proteins such as IKBKG (NEMO), TANK, or TAB2. K63 may also target proteins for the lysosome. The K63-ubiquitin chain is mediated through a cooperation of TRAF6 with E2 ubiquitin-conjugating enzymes UBE2D3 (formerly UBCH5C) or UBE2N/UBE2V1 (formerly UBC13/UEV1A) (Xu et al., 2009; Zeng et al., 2009). The M1-linkage comprises a true peptide bond between glycine-76 and αNH2 of methionine-1, it gives rise to a so-called linear chain and this plays a key role in TNF signaling (Haas et al., 2009). Linear chains are formed by the E3-ligases HOIP (RNF31), HOIL1 (RBCK1), and SHARPIN, constituting a complex that is also known as the linear ubiquitin chain assembly complex (LUBAC) (Figure 13-16) (Kirisako et al., 2006; Steiglitz et al., 2013).

The Nobel Prize in Chemistry 2004 was awarded jointly to Aaron Ciechanover, Avram Hershko, and Irwin Rose *"for the discovery of ubiquitin-mediated protein degradation."*

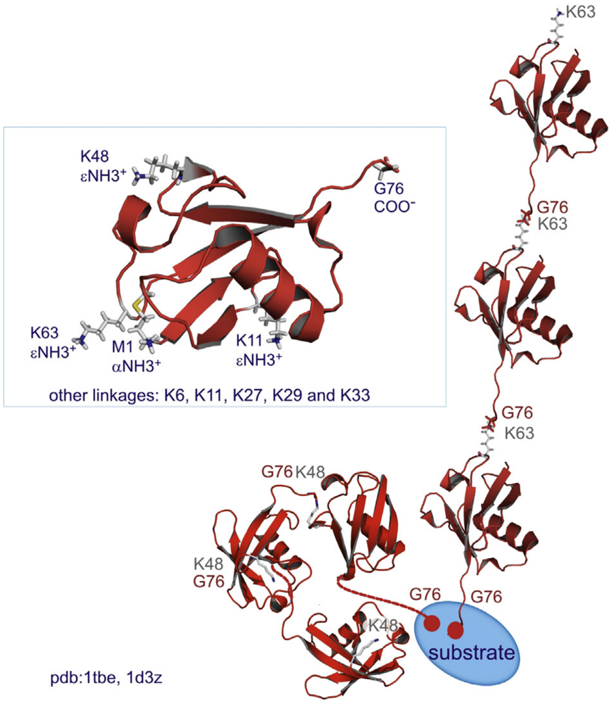

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FIGURE 13-15 Different linkages of ubiquitin chains. Ubiquitins are coupled to each other through isopeptide linkages between the εNH2 of one of seven lysines (K6, K11, K27, K29, K33, K48, or K63) and the carboxyterminal Gly76. K48-type poly-ubiquitin chains are recognized by ubiquitin-binding proteins that communicate with the proteasome. K63-ubiquitin chains play a role in the recruitment of signaling complexes (through binding of IKBKG (NEMO), TAB2, or TANK. It is also possible to link ubiquitins through the αNH2 of the initiator methionine (met-1 or M1), giving rise to a linear chain. IKBKG binds with high affinity to this type of chain.

Different classes of E3-ubiquitin ligases

While there are few E1-activating enzymes (5 genes) and a little more E2-conjugating enzymes (37 genes), there are hundreds of E3-ubiquitin protein ligases. The search string "E3-ubiquitin protein ligase" produces 383 reviewed entries in UniProt for *Homo sapiens*. Five classes of E3s are recognized, they are subdivided by one of the domains they carry: HECT-, RING-, U box-, PHD-, or an A20 Zn-finger. Among these the RING-type are most abundant. The RING domain comprises a series of Zn^{2+} finger motifs that act as a protein–protein interaction domain. Some RING-domain proteins interact with E2-ubiquitin conjugating enzymes and in the case of HOIP (RNF31) the second RING domain serves as a transitory substrate for ubiquitin. The RING-domain ligases

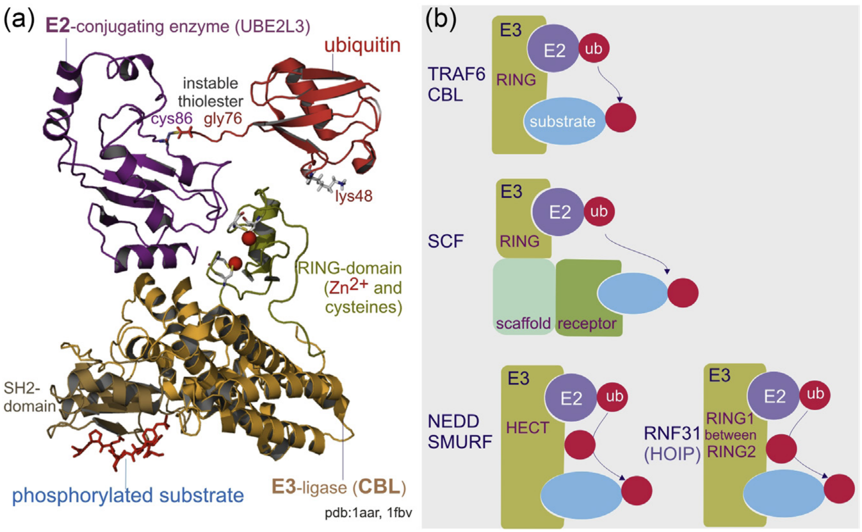

FIGURE 13-16 Mode of operation of different E3-ubiquitin protein ligases. (a) Structure of the CBL E3-ligase complex. CBL has three roles. It selects substrate, in this example, the tyrosine-phosphorylated kinase ZAP70 bound by the SH2 domain of CBL. It recruits the E2-conjugating enzyme UBE2L3 (formerly UbcH7) through its RING domain and contributes to the transfer of the glycine-76 of ubiquitin to a lysine in the substrate (not shown). Zn^{2+} ions indicated as red spots. (b) Schematic representation of the composition and mode of action of the different classes of E3-ubiquitin protein ligases. The RING ligases exist in three flavors, composed of one protein (CBL, TRAF6), or several (SCF), or carrying two RING domains of which one binds E2 and the other serves as transitory substrate for ubiquitin. HECT ligases (NEDD, SMURF) proteins are single protein-ligases and like the RING-between-RING ligases, they act as a transitory substrate (through an instable thiolester) in the transfer reaction of ubiquitin (and in essence qualify as both E2-conjugating and E3-ligase enzymes).

come in three flavors, those that possess one RING domain and act as single proteins (see Figure 13-16 for CBL) or as large complexes with scaffold proteins and a separate receptor module, and those that possess two RING domains (the RING-between-RING E3-ligases such as HOIP). In the case of the HECT and RING-between-RING ligases, the protein acts as a transitory ubiquitin substrate (exposed cysteine) before transfer to the real substrate. In Figure 13-16 we show the molecular composition of the RING-finger E3-ligase CBL to illustrate how it brings together E2-conjugating enzyme/Ubiquitin and substrate, and we provide a schematic representation of the E3-ligases discussed in this chapter (with the addition of HECT ligases to which we return in Chapter 17, “TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases”).

The RING domain motif is not restricted to E3-ubiquitin ligases. For instance, BRCA1, whose loss of function mutation is associated with breast cancer, is not involved in ubiquitinylation but uses its RING-finger motif to bind to BARD1.

SUMO and sumoylation

Overall, SUMO (small ubiquitin-like modifier) and ubiquitin share only 18% sequence identity, but the folded structures of their C-termini are almost superimposable. In vertebrates, there are three SUMO-related peptides (SUMO1-4). SUMO is implicated in a number of regulatory events such as enhancement of protein stability, protein–protein interactions, subcellular translocation, and transcriptional control (Muller et al., 2001; Johnson, 2004; Yang and Gregoire, 2006). Because ubiquitin and SUMO compete for the same target lysine-residues, sumoylation can prevent ubiquitinylation and can thus serve to protect proteins from degradation at the proteasome. The PIAS family of E3-ligases (PIAS1-4) involved in the conjugation of SUMO represent a variant on RING-type E3 ligases (Johnson, 2004). They have their own E1-activating enzymes, SAE1, UBA1-3, and UBA5-7. They are able to interact with some E2-ubiquitin-conjugating enzymes but UBE2I (formerly Ubc9) being preferred by far. Sumoylation of JUN, ELK1, MYB, and the androgen receptors (AR) by PIAS suppresses their activity (Girdwood et al., 2004). Sumoylation can also act as an activator, CREB, FOXL2, and TCF7L2 (formerly Tcf-4) and TP53 being good examples (Yamamoto et al., 2003; Comerford et al., 2003).

Ubiquitin-binding proteins

The first entity that binds ubiquitin with high specificity to be identified was the regulatory proteasome particle RAD23A (formerly HR23A). It plays a role in recognition of K48-ubiquitinylated proteins through two UBA domains (ubiquitin associated) (Deveraux et al., 1994). Another protein is PSMD4 (formerly S5a) and it harbors two ubiquitin-interacting motifs (UIM). It is a structural component of the regulatory subunit (19S) of the proteasome and one of its UIM motifs binds the ULD of RAD23A, so fixing the recognized substrate (see Figure 13-17). There are many others, of which a large proportion is involved in targeting proteins to the proteasome (Ciechanover and Stanhill, 2014; Elsasser and Finley, 2005). With respect to the recognition of K63-ubiquitin chains we have already mentioned IKBKG (NEMO), TAB2, and TANK but we can add Optineurin (OPTN), TOM1, and TOLLIP.

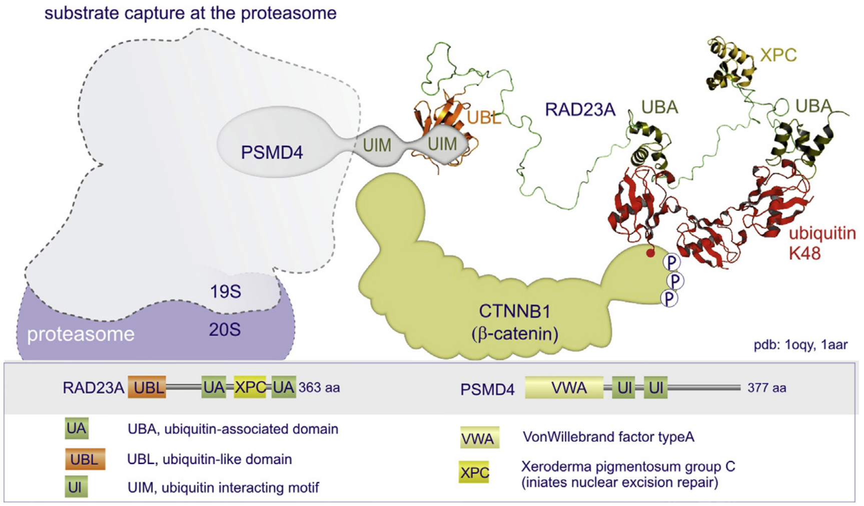
substrate capture at the proteasome
PSMD4
UIM
UIM
UBL
RAD23A
UBA
XPC
UBA
ubiquitin
K48
P
P
P
19S
20S
proteasome
CTNNB1
(β-catenin)
pdb: 1oqy, 1aar
RAD23A UBL UA XPC UA 363 aa
PSMD4 VWA UI UI 377 aa
UA UBA, ubiquitin-associated domain
UBL UBL, ubiquitin-like domain
UI UIM, ubiquitin interacting motif
VWA VonWillebrand factor typeA
XPC Xeroderma pigmentosum group C
(iniates nuclear excision repair)

FIGURE 13-17 Capturing K48-ubiquitinylated protein at the proteasome. Proteins involved in capturing substrate for the proteasome contain ubiquitin-associated (UBA) or ubiquitin-interacting motifs (UIM) (among others). Two examples are illustrated here. RAD23A is composed of two UBA sites and one ubiquitin-like domain (UBL). It binds ubiquitinylated substrate at both UBA sites, and this may be one mechanism by which only poly-ubiquitinylated proteins are selected for destruction. With its UBL domain it binds the ubiquitin interacting motif from PSMD4, an integral component of the S19 regulatory particle. The target for destruction, β-catenin, is now firmly connected to the destruction machinery.

References

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Beinke, S., Ley, S.C., 2004. Functions of NK-kB1 and NF-kB2 in immune cell biology. Biochem. J. 382, 393–409.

Belvin, M.P., Anderson, K.V., 1996. A conserved signaling pathway: the *Drosophila* toll-dorsal pathway. Annu. Rev. Cell Dev. Biol. 12, 393–416.

Beutler, B., Poltorak, A., 2001. The sole gateway to endotoxin response: how LPS was identified as Tlr4, and its role in innate immunity. Drug Metab. Dispos. 29, 474–478.

Boone, D.L., Turer, E.E., Lee, E.G., Ahmad, R.C., Wheeler, M.T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O., McNally, E., Pickart, C., Ma, A., 2004. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. Nat. Immunol. 5, 1052–1060.

Brummelkamp, T.R., Nijman, S.M., Dirac, A.M., Bernards, R., 2003. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 424, 797–801.

Chariot, A., Leonardi, A., Muller, J., Bonif, M., Brown, K., Siebenlist, U., 2002. Association of the adaptor TANK with the I kappa B kinase (IKK) regulator NEMO connects IKK complexes with IKK epsilon and TBK1 kinases. J. Biol. Chem. 277, 37029–37036.

Chen, G., Shaw, M.H., Kim, Y.G., Nunez, G., 2009. NOD-like receptors: role in innate immunity and inflammatory disease. Annu. Rev. Pathol. 4, 365–398.

Chen, Z.J., 2005. Ubiquitin signalling in the NF-kappaB pathway. Nat. Cell Biol. 7, 758–765.

Cheung, P.C., Campbell, D.G., Nebreda, A.R., Cohen, P., 2003. Feedback control of the protein kinase TAK1 by SAPK2a/p38alpha. EMBO J. 22, 5793–5805.

Ciechanover, A., Stanhill, A., 2014. The complexity of recognition of ubiquitinylated substrates by the 26S proteasome. Biochim. Biophys. Acta 1843, 86–96.

Comerford, K.M., Leonard, M.O., Karhausen, J., Carey, R., Colgan, S.P., Taylor, C.T., 2003. Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. Proc. Natl. Acad. Sci. U.S.A. 100, 986–991.

Dauphinee, S.M., Karsan, A., 2006. Lipopolysaccharide signaling in endothelial cells. Lab. Invest. 86, 9–22.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., Chen, Z.J., 2000. Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103, 351–361.

Deveraux, Q., Ustrell, V., Pickart, C., Rechsteiner, M., 1994. A 26 S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. 269, 7059–7061.

DiDonato, A., Hayakawa, M., Rothwarf, D.M., Zandi, E., Karin, M., 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388, 548–554.

Eddins, M.J., Carlile, C.M., Gomez, K.M., Pickart, C.M., Wolberger, C., 2006. Msm2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat. Struct. Mol. Biol. 13, 915–920.

Elsasser, S., Finley, D., 2005. Delivery of ubiquitinylated substrates to protein-unfolding machines. Nat. Cell Biol. 7, 742–749.

Ferrandon, D., Imler, J.L., Hoffmann, J.A., 2004. Sensing infection in *Drosophila*: toll and beyond. Semin. Immunol. 16, 43–53.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., Maniatis, T., 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. 4, 491–496.

Galloway, S.M., Raetz, C.R., 1990. A mutant of *Escherichia coli* defective in the first step of endotoxin biosynthesis. J. Biol. Chem. 265, 6394–6402.

Gioannini, T.L., Teghanemt, A., Zhang, D., Coussens, N.P., Dockstader, W., Ramaswamy, S., Weiss, J.P., 2004. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. Proc. Natl. Acad. Sci. U.S.A. 101, 4186–4191.

Girdwood, D.W., Tatham, M.H., Hay, R.T., 2004. SUMO and transcriptional regulation. Semin. Cell Dev. Biol. 15, 201–210.

Gram, H., 1884. Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. Fortschritte Med. 2, 185–189.

Guo, L., Lim, K.B., Poduje, C.M., Daniel, M., Gunn, J.S., Hackett, M., Miller, S.I., 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95, 189–198.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T., Koschny, R., Lomader, D., Silke, J., Walczak, H., 2009. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol. Cell 36, 831–844.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., Kamps, M.P., Raz, E., Wagner, H., Hacker, G., Mann, M., Karin, M., 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 439, 204–207.

Hacker, H., Tseng, P.-H., Karin, M., 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nat. Rev. Immunol. 11, 457–468.

Heguy, A., Baldari, C.T., Macchia, G., Telford, J.L., Melli, M., 1992. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the *Drosophila* toll protein are essential for IL-1R signal transduction. J. Biol. Chem. 267, 2605–2609.

Heppner, G., Weiss, D.W., 1965. High susceptibility of strain a mice to endotoxin and endotoxin-red blood cell mixtures. J. Bacteriol. 90, 696–703.

Honda, K., Taniguchi, T., 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat. Rev. Immunol. 6, 644–658.

Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., Taniguchi, T., 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 434, 772–777.

Israel, A., 2010. The IKK complex, a central regulator of NF-κB activation, Cold Spring Harb. Perspect. Biol. 2, a000158.

Janeway, C.A., 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol. Today 13, 11–16.

Johnson, E.S., 2004. Protein modification by SUMO. Annu. Rev. Biochem. 73, 355–382.

Kanayama, A., Seth, R.B., Sun, L., Ea, C.K., Hong, M., Shaito, A., Chiu, Y.H., Deng, L., Chen, Z.J., 2004. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol. Cell 15, 535–548.

Kang, Y.S., Do, Y., Lee, H.K., Park, S.H., Cheong, C., Lynch, R.M., Loeffler, J.M., Steinman, R.M., Park, C.G., 2006. A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. Cell 125, 47–58.

Kimura, Y., Tanaka, K., 2010. Regulatory mechanisms involved in the control of ubiquitin homeostasis. J. Biochem. 147, 793–798.

Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., Iwai, K., 2006. A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J. 25, 4877–4887.

Kishimoto, K., Matsumoto, K., Ninomiya-Tsuji, J., 2000. TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. J. Biol. Chem. 275, 7359–7364.

Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., Courtois, G., 2003. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitinylation. Nature 424, 801–805.

Larabi, A., Devos, J.M., Ng, S.L., Nanao, M.H., Round, A., Maniatis, T., Panne, D., 2013. Crystal structure and mechanism of activation of TANK-binding kinase 1. Cell Rep. 3, 734–746.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M., Hoffmann, J.A., 1995. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defense. Proc. Natl. Acad. Sci. U.S.A. 92, 9465–9469.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86, 973–983.

Levashina, E.A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J.A., Reichhart, J.M., 1999. Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. Science 285, 1917–1919.

Li, S., Strelow, A., Fontana, E.J., Wesche, H., 2002. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. Proc. Natl. Acad. Sci. U.S.A. 99, 5567–5572.

Lin, S.-C., Lo, Y.-C., Wu, H., 2010. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature 465, 885–890.

Liu, Y.C., 2004. Ubiquitin ligases and the immune response. Annu. Rev. Immunol. 22, 81–127.

Lohoff, M., Mak, T.W., 2005. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nat. Rev. Immunol. 5, 125–135.

Loo, Y.M., Gale, M., 2011. Immune signalling by RIG-I-like receptors. Immunity 34, 680–692.

Lu, M., Zhang, M., Takashima, A., Weiss, J., Apicella, M.A., Li, X.H., Yuan, D., Munford, R.S., 2005. Lipopolysaccharide deacylation by an endogenous lipase controls innate antibody responses to Gram-negative bacteria. Nat. Immunol. 6, 989–994.

Ma, X., Helgason, E., Phung, Q.T., Quan, C.L., Iyer, R.S., Lee, M.W., Bowman, K.K., Starovasnik, M.A., Dueber, E.C., 2012. Molecular basis of Tank-binding kinase 1 activation by trans-autophosphorylation. Proc. Natl. Acad. Sci. U.S.A. 109, 9378–9383.

Martinon, F., Burns, K., Tschopp, J., 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol. Cell 10, 417–426.

Medzhitov, R., Preston-Hurlburt, P., Janeway Jr., C.A., 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature 388, 394–397.

Mercurio, F., Manning, A.M., 1999. Multiple signals converging on NF-kappaB. Curr. Opin. Cell Biol. 11, 226–232.

Metchnikoff, E., 1884. Über eine Sprosspilzkrankheit der Daphnien. Beitrag zur Lehre über den Kampf der Phagocyten gegen Krankheitserrenger. Archiv fur Pathologische. Anat. Physiol. fur Klin. Med. 96, 177–195.

Miyamoto,M.,Fujita,T.,Kimura,Y.,Maruyama,M.,Harada,H.,Sudo,Y.,Miyata,T.,Taniguchi,T., 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 54, 903–913.

Montminy, S.W., Khan, N., McGrath, S., Walkowicz, M.J., Sharp, F., Conlon, J.E., Fukase, K., Kusumoto, S., Sweet, C., Miyake, K., Akira, S., Cotter, R.J., Goguen, J.D., Lien, E., 2006. Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. Nat. Immunol. 7, 1066–1073.

Moore, K.W., de Waal, M.R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765.

Morisato, D., Anderson, K.V., 1995. Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annu. Rev. Genet. 29, 371–399.

Muller, S., Hoege, C., Pyrowolakis, G., Jentsch, S., 2001. SUMO, ubiquitin's mysterious cousin. Nat. Rev. Mol. Cell Biol. 2, 202–210.

Munford, R.S., Varley, A.W., 2006. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog. 2, e67.

Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dirac, A.M., Sixma, T.K., Bernards, R., 2005. A genomic and functional inventory of deubiquitinating enzymes. Cell 123, 773–786.

Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., Matsumoto, K., 1999. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 398, 252–256.

Nusslein-Volhard, C., Lohs-Schardin, M., Sander, K., Cremer, C., 1980. A dorso-ventral shift of embryonic primordia in a new maternal-effect mutant of *Drosophila*. Nature 283, 474–476.

O'Neill, L.A., Golenbock, D., Bowie, A.G., 2013. The history of Toll-like receptors-redefining innate immunity. Nat. Rev. Immunol. 13, 453–460.

Panne, D., Maniatis, T., Harrison, S.C., 2007. An atomic model of the interferon-β enhanceosome. Cell 129, 1111–1123.

Picard, C., Puel, A., Bonnet, M., Ku, C.L., Bustamante, J., Yang, K., Soudais, C., Dupuis, S., Feinberg, J., Fieschi, C., Elbim, C., Hitchcock, R., Lammas, D., Davies, G., Al-Ghonaium, A., Al-Rayes, H., Al-Jumaah, S., Al-Hajjar, S., Al-Mohsen, I.Z., Frayha, H.H., Rucker, R., Hawn, T.R., Aderem, A., Tufenkeji, H., Haraguchi, S., Day, N.K., Good, R.A., Gougerot-Pocidalo, M.A., Ozinsky, A., Casanova, J.L., 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science 299, 2076–2079.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–2088.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., Bazan, J.F., 1998. A family of human receptors structurally related to *Drosophila* Toll. Proc. Natl. Acad. Sci. U.S.A. 95, 588–593.

Sano, Y., Harada, J., Tashiro, S., Gotoh-Mandeville, R., Maekawa, T., Ishii, S., 1999. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. J. Biol. Chem. 274, 8949–8957.

Schroder, N.W., Schumann, R.R., 2005. Non-LPS targets and actions of LPS binding protein (LBP). J. Endotoxin Res. 11, 237–242.

Schumann, R.R., Belka, C., Reuter, D., Lamping, N., Kirschning, C.J., Weber, J.R., Pfeil, D., 1998. Lipopolysaccharide activates caspase-1 (interleukin-1-converting enzyme) in cultured monocytic and endothelial cells. Blood 91, 577–584.

Sen, R., Baltimore, D., 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46, 705–716.

Shen, D.D., Hahn, W.C., 2011. Emerging roles for the non-canonical IKKs in cancer. Oncogene 30, 631–641.

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., Shao, F., 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. Nature 514, 187–192.

Shu, C., Sankaran, B., Chaton, C.T., Herr, A.B., Mishra, A., Peng, J., Li, P., 2013. Structural insights into the functions of TBK1 in innate antimicrobial immunity. Structure 21, 1137–1148.

Siebenlist, U., Franzoso, G., Brown, K., 1994. Structure, regulation and function of NF-kappa B. Annu. Rev. Cell Biol. 10, 405–455.

Sims, J.E., Acres, R.B., Grubin, C.E., McMahan, C.J., Wignall, J.M., March, C.J., Dower, S.K., 1989. Cloning the interleukin 1 receptor from human T cells. Proc. Natl. Acad. Sci. U.S.A. 86, 8946–8950.

Steiglitz, B., Rana, R.R., Koliopoulos, M.G., Morris-Davies, A.C., Schaeffer, V., Christodoulou, E., Howell, S., Brown, N.R., Dikic, I., Rittinger, K., 2013. Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP. Nature 503, 422–426.

Steiner, A.A., Chakravarty, S., Rudaya, A.Y., Herkenham, M., Romanovsky, A.A., 2006. Bacterial lipopolysaccharide fever is initiated via toll-like receptor 4 on hematopoietic cells. Blood 107, 4000–4002.

Steinman, R.M., Cohn, Z.A., 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J. Exp. Med. 137, 1142–1162.

Sun, S.C., 2011. Non-canonical NFkB signaling pathway. Cell Res. 21, 71–85.

Suzuki, N., Suzuki, S., Duncan, G.S., Millar, D.G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J.M., Wesche, H., Ohashi, P.S., Mak, T.W., Yeh, W.C., 2002. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature 416, 750–756.

Takaesu, G., Kishida, S., Hiyama, A.K., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., Matsumoto, K., 2000. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol. Cell 5, 649–658.

Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T.W., Taniguchi, T., 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 434, 243–249.

Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., Mosialos, G., 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 14 (424), 793–796.

Tu, D., Zhu, Z., Zhou, A.Y., Yun, C.H., Lee, K.E., Toms, A.V., Li, Y., Dunn, G.P., Chan, E., Thai, T., Yang, S., Ficarro, S.B., Marto, J.A., Jeon, H., Hahn, W.C., Barbie, D.A., Eck, M.J., 2013. Structure and ubiquitinylation-dependent activation of TANK-binding kinase 1. Cell Rep. 3, 747–758.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., Chen, Z.J., 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412, 346–351.

Weber, A.N., Tauszig-Delamasure, S., Hoffmann, J.A., Lelievre, E., Gascan, H., Ray, K.P., Morse, M.A., Imler, J.L., Gay, N.J., 2003. Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling. Nat. Immunol. 4, 794–800.

Wertz, I.E., 2011. It takes two to tango: a new couple in the family of ubiquitin-editing complexes. Nature 12, 1133–1135.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433.

Wu, C.J., Conze, D.B., Li, T., Srinivasula, S.M., Ashwell, J.D., 2006. Sensing of Lys 63-linked polyubiquitinylation by NEMO is a key event in NF-kappaB activation [corrected]. Nat. Cell Biol. 8, 398–406.

Xiao, G., Harhaj, E.W., Sun, S.C., 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol. Cell 7, 401–409.

Xu, M., Skaug, B., Zeng, W., Chen, Z.J., 2009. A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFα and IL-1β. Mol. Cell 36, 302–314.

Yamamoto, H., Ihara, M., Matsuura, Y., Kikuchi, A., 2003. Sumoylation is involved in beta-catenin-dependent activation of Tcf-4. EMBO J. 22, 2047–2059.

Yang, X.J., Gregoire, S., 2006. A recurrent phospho-sumoyl switch in transcriptional repression and beyond. Mol. Cell 23, 779–786.

Yin, Q., Lin, S.-C., Lamothe, B., Lu, M., Lo, Y.-C., Hura, G., Zhen, L., Rich, R.L., Campos, A.D., Myszka, D.G., Lenardo, M.J., Darnay, B.G., Wu, H., 2009. E2 interaction and dimerization in the crystal structure of TRAF6. Nat. Struc. Mol. Biol. 16, 658–666.

Zeng, W., Xu, M., Liu, S., Sun, L., Chen, Z.J., 2009. Key role of Ubc5 and lysine-63 polyubiquitinylation in viral activation of IRF3. Mol. Cell 36, 315–325.

CHAPTER

14

Chemokines and Traffic of White Blood Cells

INFLAMMATION AND LEUKOCYTES

The first indications that white cells, under conditions of inflammation, adhere to the walls of the finer vessels and then emigrate into the tissues were recorded more than 150 years ago (Figure 14-1) (Addison, 1843; Waller, 1846a,b). Cohnheim's detailed histological description of 1882 (Cohnheim, 1882; Del Maschio et al., 1999) remained the basis of most textbook accounts of inflammation for a further 80 years. Later on, the first electron microscopic investigation of leukocyte adherence and migration provided much needed detail but failed to reveal how cells adhere to the inflamed endothelium (Marchesi and Florey, 1960). Nor were any ideas forthcoming about how the cells penetrate an apparently coherent endothelial cell layer.

In bacteria, the initiating amino acid is formylmethionine, fMet (in eukaryotes, it is methionine except for mitochondria). Synthetic peptides containing the sequence fMetXY (X and Y are hydrophobic amino acids) act as powerful stimulants for neutrophil activation: among the most potent is fMetLeuPhe (Toniolo et al., 1984). fMet peptides isolated from bacterial cultures also activate neutrophils (Bennett et al., 2006). Collectively, these have been called chemotactic peptides although they act to stimulate all the multiple functions of these cells such as the respiratory burst and secretion of lysosomal enzymes in addition to chemotaxis.

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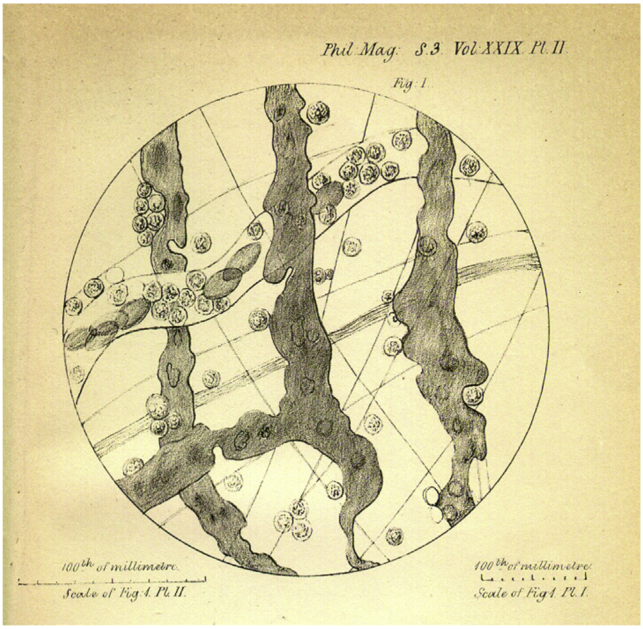


FIGURE 14-1 Augustus Waller's microscopic examination of cell adherence and extravasation from the vessels of an inflamed frog tongue. "The blood, as we are aware, consists of a transparent fluid holding in suspension numerous particles, most of which are red and of a flattened shape, while a few others are colorless, and spherical in form … The peculiar manner in which the lymph-globules, or corpuscles, conduct themselves when in the capillaries, when in an organ in a state of irritation, has of late engaged much attention. The experiments of Mr W Addison of Malvern, have greatly contributed to show these important functions in inflammation. In the tongue of the frog and toad they may be frequently seen circulating with the red particles in the vessels, down to the minutest capillaries. As it has already been pointed out, these spherules are generally found, when they come into contact with the parieties of the vessels, to retain their adherence with greater force than is manifested by the red particles in the like circumstances; as in the figure, where the current was observed to continue for many minutes without displacing the globules near the sides of the vessel. Thus we frequently see a lymph-globule remain in the same place, notwithstanding the current of red particles sweeping and pushing by it. The appearance in the larger vessels of these spherules, adherent to their inner surface, has been very aptly compared to so many pebbles or marbles over which a stream runs without disturbing them … The corpuscles, which are transparent, are occasionally seen to be granulated … Let us now examine the admirable manner in which nature has solved the apparent paradox, of eliminating, from a fluid circulating in closed tubes, certain particles floating in it, without causing any rupture or perforation in the tubes, or allowing the escape of the red particles, which are frequently the smaller of the two, or that of the fluid part of the blood itself … After the observation had continued for half an hour, numerous corpuscles were seen outside the vessels, together with a very few blood discs in the proportion of about one to ten of the former. No appearance of rupture could be seen in any of the vessels. The corpuscles were generally distant about 0.03 mm from their parieties. After the experiment had lasted about two hours, thousands of these corpuscles were seen scattered over the membrane, with scarcely any blood discs … No trace of the corpuscular extravasation could be seen, except the presence of the corpuscles themselves … I consider therefore as established, 1st, the passage of these corpuscles 'de toute pièce' through the capillaries; 2ndly, the restorative power in the blood, which immediately closes the aperture thus formed … In endeavoring to account for the fact of the passage of the corpuscles through the vessels we find considerable difficulties. It cannot be referred to the influence of vitality, as it is observed likewise to take place after death. It may be surmised, either that the corpuscle, after remaining a certain time in contact with the vessel, gives off by exudation from within itself some substance possessing a solvent power over the vessel, or that the solution of the vessel takes place in virtue of some of those molecular actions which arise from the contact of two bodies; actions which are now known as exerting such extensive influence in digestion, and are referred to what is termed the catalytic power." *Paraphrased from Waller (1846a,b).*

INFLAMMATORY MEDIATORS

It is essential that migratory cells such as leukocytes can tolerate changes in their environments. They must be able to move from the bone marrow (where they are attached) into the blood and then into the tissues, particularly the lymph nodes (where they become attached again). To do all this, they must be able to switch their integrins "on" and "off." If the integrins on leukocytes were left permanently "on," they could never leave the bone marrow. Once in the blood, the integrin function should be switched "off." Leukocytes leave the circulation when they encounter an appropriate signal of inflammation or infection or more generally in the continuous process of immune surveillance. For monocytes and neutrophils, departure from the circulation is a one-way journey, their destiny with death in the tissues. By contrast, lymphocytes move from the blood into the tissues, then through the lymphatic system, and then back into blood. We focus on the regulation of leukocyte adhesion and extravasation under inflammatory conditions (Figure 14-2).

Protein A. Derived from *Staphylococcus aureus* (SpA), protein A binds with very high affinity to both the constant (Fcγ) and variable (Fab) regions of IgG and IgM antibodies (Graille et al., 2000). This has been successfully exploited in immunoprecipitation protocols using protein A coupled to Sepharose beads. Protein A contributes to the pathology of *S. aureus* by directly binding to TNF receptors on the respiratory epithelium. This plays an important role in the onset of inflammation of the lower respiratory tract leading to severe pneumonia associated with tissue damage and sometimes sepsis (Gomez et al., 2004). Protein A can also bind some membrane-associated B-cell antigen receptors and promote antibody production (Kristiansen et al., 1994). This plays a role in the defense against the pathogens and is also associated with autoimmune disorders such as rheumatoid arthritis.

At sites of inflammation (caused by injury or infection), mediators are released that affect the expression and affinity of adhesion molecules and that affect the release of chemokines from endothelial cells. Lipopolysaccharide (LPS), acting through its receptor TLR4 (Toll-like receptor: return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation"), induces the expression of inflammatory mediators including cytokines and chemokines. Other bacterial and cellular components (listed in Figure 14-2 and

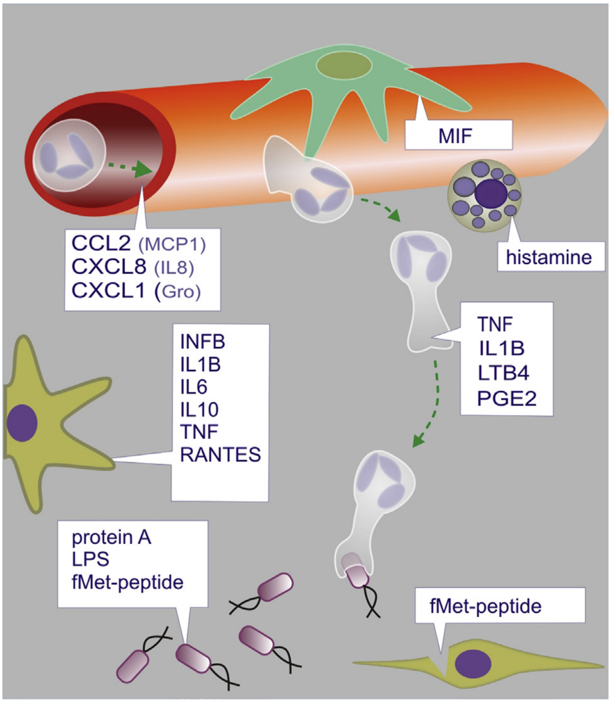


FIGURE 14-2 Generation of inflammatory mediators at sites of infection. Tissue damage and bacterial infection induce the release of inflammatory mediators from various cell types. Bacterial protein A binds and activates the TNF receptor. Bacterial lipopolysaccharide (LPS), which acts on local fibroblasts, dendritic cells, vascular endothelial cells, mast cells, and resident leukocytes, induces the release of histamine, INFβ, IL1β, IL6 RANTES, and TNF, as well as the chemokines CXCL1 (Gro), CXCL8 (IL8), and CCL2 (MCP1). Pericytes (green) release macrophage migration inhibitory factor (MIF). Collectively, these mediators are responsible for the upregulation of adhesion molecules on vascular endothelial cells and for the activation of integrins on leukocytes. This results in their extravasation followed by migration to sites of damage/infection. Formylmethionyl peptides released from bacteria (as well as from the damaged mitochondria of host cells) facilitate the migratory response (chemotaxis). These processes allow the rapid accumulation of leukocytes, in particular neutrophils, at sites of infection as a first line of defense. It also initiates the process of tissue remodeling and repair (wound healing).

in Table 14-1) can be added to the list of “patterns” that provoke the release of inflammatory mediators. One of these is protein A, a staphylococcal surface protein that binds to the TNF receptor. Another is the class of formylmethionyl peptides, generated as the initiation sequences of prokaryotic proteins and that are released by microbes. Histamine, released from mast cells and basophils, plays an important role in locally widening the vasculature, therefore allowing accumulation of blood and thus the accumulation of leukocytes at the site of inflammation. Pericytes attached to the vascular bed produce macrophage migration inhibitory factor (MIF), offering the freshly entered blood-borne cells a new set of instructions.

TABLE 14-1 Sources of inflammatory mediators

source	Example
Release from invading microorganisms	Lipopolysaccharide (LPS or endotoxin) shed from the surface. Also N-formylmethionyl peptides (fMet peptides) derived from the initiator sequence of bacterial protein synthesis and protein A, a cell surface protein of *Staphylococcus aureus* that binds and activates the TNF receptor-1.
Release from resident cells	TNF and IL1B from activated macrophages or fibroblasts, histamine from mast cells, and chemokines (CCL2, CXCL8, CXCL1, formerly MCP1, IL-8, Gro) from endothelial cells or resident leukocytes.
Release from the mitochondria of damaged cells (Shawar, Rich, Becker)	Peptides derived from the amino-terminus of proteins (such as NAD^+ dehydrogenase) coded by the mitochondrial genome. These share with eubacteria the characteristic *N*-formylmethionyl initiator sequence.
Components of complement and of fibrinolysis	C5a and thrombin, respectively.

Before all this happens, vascular endothelial cells have to be prepared. In the next sections, we describe the signaling pathway induced by tissue necrosis factor, an essential cytokine that signals the onset of the inflammatory response (and thus of innate immunity). Its presence results in an elevated cell surface expression of adhesion molecules and the release of yet more inflammatory mediators from vascular endothelial cells. It also plays a crucial role in regulating the extravasation of circulating leukocytes (also known as transendothelial cell migration or diapedesis).

TUMOR NECROSIS FACTOR: POTENTIAL ANTITUMOR AGENT OR INFLAMMATORY CYTOKINE?

The observation that the size of a tumor occasionally diminishes after bacterial infection has a long history. As early as 1848, LeGrand noted two cases of longstanding scrofulous lymphoma that appeared to regress after infection with erysipelas. In 1888, Bruns reported on the spontaneous regression of human tumors following infection with *S. erysipelas* (Bruns, 1888):

> The frequently observable fact that new formations, particularly those of a malign nature may be caused to regress or to disappear by a concurrent erysipelas is of very great interest both in theory and practice. In respect of the former this fact merits particular attention, especially at the present time, given the current preoccupation with the study of the aetiology of new formations, the results of which may well be capable of shedding new light upon the beneficial effects of erysipelas. On the practical front these observations have given rise to experiments in which curative erysipelas is

> artificially induced in order to bring about the healing of inoperable new formations. However the admissibility of such experiments remains an open question …

Erysipelas: An acute superficial form of cellulitis involving the dermal lymphatics, usually caused by infection with group A streptococci. Formerly called St Anthony's fire.

The principle of bacterial infection and immune stimulation as a component in the armory of antitumor medicine persists today. Lovaxin C and other vaccines based on genus *Listeria* are being tested for therapeutic purpose in cervical, breast, ovarian, and lung cancers. In animal models of breast cancer, the Lovaxin B vaccine, composed of *Listeria* with the HER2/Neu antigen, appears to stop tumor growth (Paterson and Maciag, 2005). Activation of the immune system not only by bacterial infection but also by blocking apoptosis of T cells with an antibody against PDCD1 (nivolumab) overcomes immune resistance of melanoma and nonsmall-cell lung cancers (Topolian et al., 2012).

ORIGINAL TEXT

> Die öfters beobachtete Thatsache, dass Neubildungen, namentlich malingner Natur, durch ein interkurrentes Erysipel zur Verkleinerung oder zum Verschwinden gebracht werden, ist von hervorragendem theoretischem und praktischem Interesse. In ersterer Hinsicht verdient diese Thatsache gerade gegenwärtig besondere Beachtung, wo die Untersuchung der Aetiologie der malignen Neubildungen auf der Tagesordnung steht, deren Ergebnisse vielleicht geeignet sind, auch über die Art und Weise der salutären Wirkung des Erysipels Licht zu verbreiten. In praktischer Beziehung haben jene Beobachtungen zu Versuchen mit der kunstlichen Erzeugung eines kurativen Erysipels Veranlassung gegeben, um inoperable bösartige Neubildungen zur Heilung zu bringen.
>
> Allein die Zulässichkeit solcher Versuche ist noch eine offene Frage…:

Coley (1891) reported several cases of tumor (new growth) regression and even disappearance following repeated inoculation of erysipelas. Speculating on possible mechanisms, he considered:

> (1) that the erysipelas coccus has a direct destructive action upon the cell elements of the new growth; (2) that the high temperature (induced by the infection) alone is sufficient; (3) that sarcoma and carcinoma are both of bacterial origin and the

> erysipelas germ has a direct antagonistic effect upon the cancer bacillus … it seems in the light of present knowledge not improbable that all of the three theories may contain an element of truth, and that a larger theory combining all these elements is necessary to explain the curative action of erysipelas.

In the firm belief that the cure of cancer was at hand, Coley and others applied cell-free filtrates of streptococci as "Coley's toxins" over a period of about 40 years, apparently with some success (Coley, 1891; Pearl, 1929; Nauts et al., 1953). For a brief historical review of the application of Coley's toxins in the treatment of bone and soft-tissue sarcomas, see McCarthy (2006). The effect of these bacterial products is associated with severe hemorrhagic reaction mainly confined to the core of the tumor, which rapidly sloughs off. However, it generally leaves a ring of viable tissue that, unfortunately, continues to grow. Not surprisingly, such treatments also tend to cause widely disseminated systemic effects, and all too frequently, these can lead to circulatory collapse and death. Early attempts to separate the hemorrhagic from the cytotoxic components in filtrates of cultured *Serratia marcescens* or *Escherichia coli* (both Gram negative) led to the isolation of LPS (or endotoxin). For more detail about this molecule, return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation," Section "Lipopolysaccharides: Shield and Signal") (Shear and Andervont, 1936; Shear and Turner, 1943). Among its many biological activities, this could elicit hemorrhagic necrosis of both experimental and primary subcutaneous tumors (Anderson and Legallaies, 1952). All this provoked the hunt for immune mediators, cytotoxic for transformed cells but not causing septic shock (Carswell et al., 1975). The first of these, the TNF-α (gene name TNF) and TNF-β (lymphotoxin-alpha or LTA), attracted immense interest (Kolb and Granger, 1968; Ruddle and Waksman, 1968). However, TNF was also found to possess general pro-inflammatory properties and provoke severe systemic toxicity. The focus of interest shifted from tumor necrosis to mechanisms of inflammation.

TNF AS TARGET FOR ANTI-INFLAMMATORY DRUGS

An excess of TNF is associated with chronic inflammatory disorders. The best studied in this respect are Crohn's disease (destructive inflammation of the bowel) and rheumatoid arthritis (destructive inflammation of the synovial joints). TNF is the key cytokine-regulating expression of all other inflammatory cytokines. It was concluded that by blocking its action it would be possible to reduce the general state of inflammation

continued

TNF AS TARGET FOR ANTI-INFLAMMATORY DRUGS *(cont'd)*

(Feldmann et al., 1992). Two approaches to lower the TNF concentration have been exploited successfully. One uses a humanized antibody against TNF (Infliximab) while the other uses an extracellular fragment of the TNFRSF1B coupled to IgG1 (Etanercept). Both these treatments provide considerable relief (Feldmann and Maini, 2001; Ostermann et al., 2002). More recently, the pre-ligand assembly domains of the TNFRSF1A and TNFRSF1B have been targeted by a soluble cysteine-rich region to prevent homophilic trimerization of the receptors. This too reduces the symptoms of arthritis in animal models (Deng et al., 2005).

THE FAMILY OF TNF PROTEINS AND RECEPTORS

TNF and LTA are members of a large family of cytokines (Aggarwal, 2003). They are initially generated in a precursor form, as transmembrane proteins are arranged as stable homotrimers. From these, soluble homotrimeric cytokines are released by the action of membrane-bound metalloproteases. With one or two exceptions, these ligands bind their receptors both in a membrane-bound and in soluble states. With respect to the exceptions, an example in this case is FASLG (CD95L). As a membrane protein, this causes apoptosis of cells bearing its receptor (FAS), whereas when soluble, it prevents apoptosis (Suda et al., 1997). Another example is TNF, which only effectively activates the TNFRSF1B in its membrane form (memTNF) but not in its soluble form (sTNF) (Grell et al., 1995).

Web resource

For more information about the members of the TNF ligand and receptor superfamily (TNFRSF), consult the HUGO gene name database. URL: http://www.genenames.org/cgi-bin/genefamilies/set/781 and http://www.genenames.org/cgi-bin/genefamilies/set/782.

The TNF ligands signal through 29 different receptors, members of the TNFR superfamily (TNFRSF) (examples in Figure 14-3). These receptors can be divided into two groups: those having a death domain (DD, e.g., TNFRSF1A, ubiquitously expressed) and those without (e.g., TNFRSF1B), typically found in cells of the immune system and whose expression level is context dependent. Members of the first group interact with DD-possessing

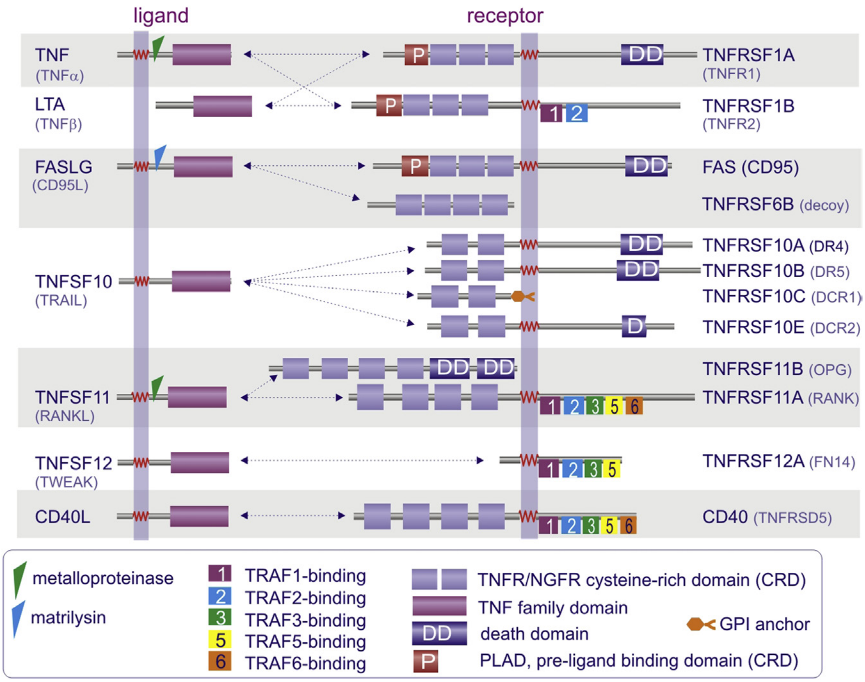


FIGURE 14-3 Domain architecture of some of the members of the TNFR superfamily. The C-terminal segments of the ligands, carrying the TNF-homology domain, have 20–30% sequence identity and bind the receptor. TNF and TNSF11 (RANKL) are released by metalloproteases of the ADAM family, whereas FASLG (CD95L) is released by matrilysin at the plasma membrane. The receptors are transmembrane proteins, except for TNFRSF10C (DCR1), which possess an inositol-lipid anchor. Note that certain receptors carry a death domain allowing them to recruit adapters/effectors with the same domain (homotypic interaction), whereas others have short motifs that recruit different members of the TRAF family (E3-ubiquitin protein ligases). *Figure adapted from Aggarwal (2003).*

proteins and may couple to a caspase-activating pathway leading to cell death. The second group has the capacity to interact directly with the effector TRAF. The possession of a DD, however, does not prevent the receptor from signaling to pathways that have little to do with cell death. In fact, one of these pathways mediates the activation of NFKB and constitutes an important cell survival signal (Tamatani et al., 1999). And conversely, death can be induced by receptors of the TNFR2 family that lack DDs.

Although macabre names have been bestowed on some of the TNF-receptor signaling components, TNF itself is better considered as a major inflammatory cytokine having the ability to induce cell death either through the process of apoptosis or through necroptosis as an option (Wajant et al., 2003; Vandenabeele et al., 2010). Both apoptosis and necroptosis may either aggravate or diminish the inflammatory state, depending on the type of cell, animal, or experimental condition. Generally, TNFRSF1A is the key mediator of TNF signaling. In endothelial cells, binding of TNF causes enhanced expression of Selectins, VCAM1, and members of the ICAM adhesion molecules (Osborn et al., 1989). It also causes the release of chemokines (CXCL1, CXCL8, CCL2) and cytokines (CSF2, IL1β, IL6, TNF) and also the expression of the enzymes NOS2, PTGS2, and cytosolic PLA2G6. All these effects of TNF and those induced by other inflammatory stimuli are mediated through the nuclear transcription factor NFKB (nuclear factor κB), a protein complex that attaches to the κB enhancer region (Sen and Baltimore, 1986). NFKB collaborates with proteins that constitute the AP1 complex such as ATF2 and JUN and with interferon regulatory factors (IRF); return to Chapter 13, "Activation of the Innate Immune System," Figure 13-5. As a result of all this, the adherence of leukocytes to the vascular wall is greatly enhanced (Pohlman et al., 1986). Later we describe the process in more detail.

Two alternative signaling events may arise downstream of the TNF receptor, both giving rise to cell death: one leading to the activation of caspases causing apoptosis, and the other leading to the activation of mixed lineage protein kinase L and resulting in necroptosis (nonaccidental necrosis) (Figure 14-4). These pathways are not further discussed.

TNF AND REGULATION OF ADHESION MOLECULE EXPRESSION IN ENDOTHELIAL CELLS

Receptor activation and formation of complex I

In the absence of ligand, the N-terminal cysteine-rich domains of the TNFRSF1A interact with each other, maintaining the silent state (Figure 14-4). Binding to the trimeric ligand, TNF, then induces either an activating conformational change or allows the formation of higher order complexes that bestow signal competence. Non-engaged receptors interact with a signal-silencing protein, BAG4 (formerly, SODD, silencer of DD). When overexpressed, this blocks the NFKB pathway as described later (Takada et al., 2003). Activation of the TNFRSF1A receptor causes BAG4 to dissociate and initiates the formation of a signaling complex on the basis of homophilic DD interactions. The DD was first identified as a stretch of 80 amino acids in FAS (CD95) necessary for the induction of programmed cell death (apoptosis) (Itoh and Nagata, 1993). Through the DD domain, the TNFR1 recruits TRADD and RIPK1.

TRADD is an adaptor protein that is complexed with TRAF2 (Hsu et al., 1995; Park et al., 2000). TRAF2 contains two interaction domains, DD and TRAF-C. With the TRAF-C domain, it binds the N-terminal domain of TRADD. The TRAF-C domain also binds TNF receptors that lack the DD such as TNFRSF1B (Figure 14-3), but it offers the opportunity to downstream signaling components such as RIPK1 or the MAP3

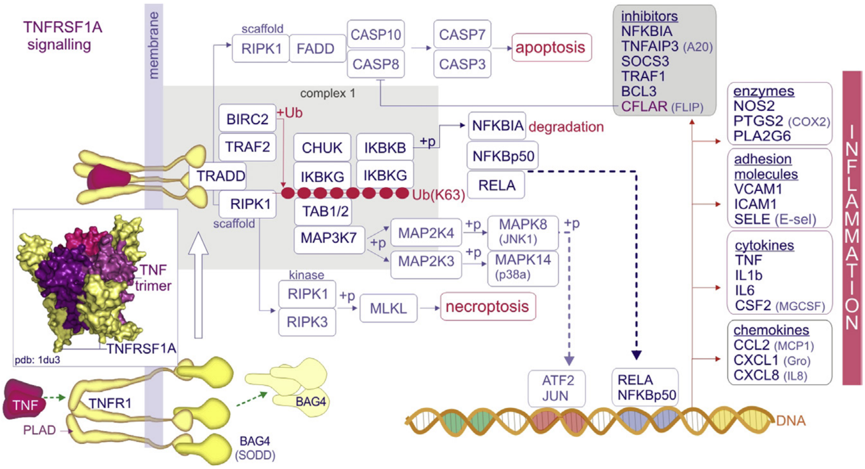


FIGURE 14-4 Global overview of the TNF signaling pathways. In the absence of ligand, TNF receptors are associated through their N-terminal pre-ligand assembly domain (PLAD). The intracellular segment is bound to BAG4 (formerly SODD for silencer of death domains). PLAD and SODD control the silencing of the unoccupied receptor. Binding of ligand induces trimerization of the receptors, and this reveals death domain-docking sites. Death domain-carrying proteins such as TRADD and RIPK1 associate with the receptors. A cascade of further recruitment ensues leading to BIRC2-mediated ubiquitinylation of RIPK1. The K63-linked ubiquitin chain attracts IKBKG (NEMO) and TAB2, themselves associated with protein kinases, CHUK (IKKα), IKBKB (IKKβ), and MAP3K7 (TAK1). Altogether they are referred to as “complex I.” Complex I causes the expression of a long list of genes that are involved in inflammation, among which adhesion molecules and chemokines are the subject of this chapter. Note that this signaling pathway also induces the expression of CFLAR (FLIP), an essential inhibitor of apoptosis. The insert shows the structure of TNFRSF10A (DR4) bound to TNFSF10 (TRAIL). Three receptor molecules bind a trimeric ligand. Failure to constitute a stable complex I opens the way to two alternative pathways (formation of two different type-II complexes). One results in apoptosis through the activation of initiator caspase-8 and caspase-10, as a consequence of the formation of an RIPK1/FADD scaffold (death-inducing signaling complex or DISC). The other causes the activation of the mixed lineage protein kinase MLKL as a result of the combined kinase activity of RIPK1/RIPK3, and this leads to necroptosis (nonaccidental necrosis).

kinases, MAP3K5 (ASK1), and MAP3K1 (MEKK1). Moreover, TRAF2 contains a RING Zn-finger and multiple TRAF Zn-finger motifs, so qualifying it as an E3-ubiquitin ligase. However, despite the possession of a RING finger, TRAF2 serves primarily as a scaffold protein and as a substrate of other E3-ubiquitin protein ligases. In the context of TNF signaling, it fails to manifest E3-ligase activity. TRAFs form homotrimers through their coiled-coil motif (for clarity reasons not generally shown in images).

RIPK1 too has a DD at the C-terminus, and it possesses an N-terminal serine/threonine kinase domain (Ea et al., 2006). The protein carries six lysines accessible for ubiquitinylation. Kinase activity is not essential for signaling through complex I nor is it of importance in the process of activation of caspases (apoptosis pathway). However, it is essential for the process of necroptosis. In the context of NFKB signaling, such as TRAF2, RIPK1 is best described as a scaffold protein and a substrate for E3-ubiquitin protein ligases. Return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signaling through Ubiquitinylation," Figure 13-6 for an impression of how trimerized TNF receptors may recruit DD-containing proteins.

In the Prosite database, the TRAF-C domain is referred to as MATH domains because of its resemblance to Meprin (Meprin and Traf homology).

Importantly, the coiled-coil region of TRAF2 binds the E3-ubiquitin protein ligase BIRC2 (baculovirus IAP repeat-containing protein-2, formerly cIAP). Its RING finger binds several E2-ubiquitin conjugating enzymes, among which UBE2D3 (formerly UBC5C). Figure 14-5 illustrates the domain architecture of the aforementioned proteins and the way they interact with each other. The sequence of events is depicted in Figure 14-6.

BIRC2 associated with UBE2D3 makes ubiquitin chains by connecting Lys-63 with Gly-76, the so-called K63-linkage giving rise to K63-ubiquitin chains. For more information about the ubiquitinylation process and the different linkages, return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signaling through Ubiquitinylation," Section "Essay: ubiquitinylation and SUMOylation." The main substrate of BIRC2 is RIPK1. The growing K63-ubiquitin chain serves as a docking site for IKBKG (NEMO) (Ngadjeua et al., 2013) and TAB2 (Kulathu et al., 2009). These proteins bind at the interface of two ubiquitins and are therefore sensitive to the type of linkage (insert in Figure 14-6). They bring into the complex the protein kinases MAP3K7 (TAK1), CHUK (IKKα), and IKBKB (IKKβ) (inhibitor of NF-kappa-B kinase-alpha and -beta) into the complex. This leads to their activation in a process that requires both transphosphorylation, MAP3K7 phosphorylates IKBKB on residue Ser-177, and autophosphorylation, IKBKB phosphorylates itself on Ser-181. Both serine residues situate in the activation segment, and their phosphorylation renders the protein kinase competent (Zhang et al., 2014). Importantly, IKBKB carries the signal further down the pathway. The assembled adaptors, scaffolds, and kinases constitute the TNF–receptor complex I (return to Figure 14-4).

Signaling through NFKB

The NFKB transcription factor complex, comprising NFKBp50 and RELA, is attached to a protein, NFKBIA (for NFKB-inhibitor protein alpha),

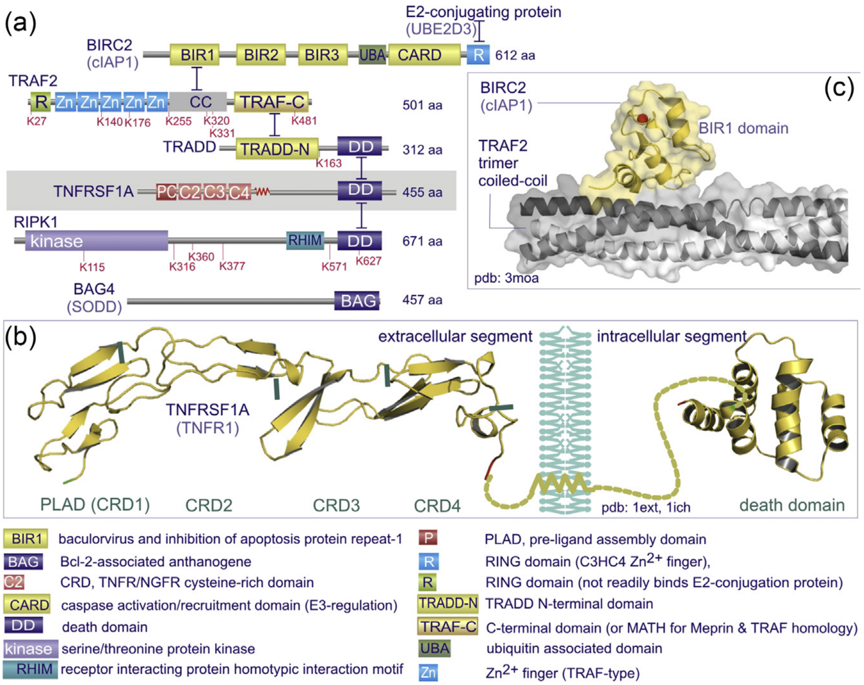

(a)
E2-conjugating protein
(UBE2D3)
BIRC2
(cIAP1)
BIR1
BIR2
BIR3
UBA
CARD
R
612 aa
TRAF2
R
Zn
Zn
Zn
Zn
Zn
CC
TRAF-C
K27
K140
K176
K255
K320
K331
K481
501 aa
TRADD
TRADD-N
DD
K163
312 aa
TNFRSF1A
P
C2
C3
C4
DD
455 aa
RIPK1
kinase
RHIM
DD
K115
K316
K360
K377
K571
K627
671 aa
BAG4
(SODD)
BAG
457 aa
(c)
BIRC2
(cIAP1)
BIR1 domain
TRAF2
trimer
coiled-coil
pdb: 3moa
(b)
extracellular segment
intracellular segment
TNFRSF1A
(TNFR1)
PLAD (CRD1)
CRD2
CRD3
CRD4
pdb: 1ext, 1ich
death domain
BIR1 baculorvirus and inhibition of apoptosis protein repeat-1
BAG Bcl-2-associated anthanogene
C2 CRD, TNFR/NGFR cysteine-rich domain
CARD caspase activation/recruitment domain (E3-regulation)
DD death domain
kinase serine/threonine protein kinase
RHIM receptor interacting protein homotypic interaction motif
P PLAD, pre-ligand assembly domain
R RING domain (C3HC4 Zn2+ finger),
R RING domain (not readily binds E2-conjugation protein)
TRADD-N TRADD N-terminal domain
TRAF-C C-terminal domain (or MATH for Meprin & TRAF homology)
UBA ubiquitin associated domain
Zn Zn2+ finger (TRAF-type)

FIGURE 14-5 Domain architecture of proteins that associate with the TNF receptor. (a) The death domain of the TNF receptor interacts with the death domain of RIPK1 and TRADD. RIPK1 is a protein kinase, but in this context it acts as a scaffold protein and a ubiquitin substrate. TRADD in turn binds with its N-terminal domain to the TRAF-C domain of TRAF2. Although TRAF2 contains a RING finger and belongs to a family of E3-ubiquitin ligases, it appears not to be functional and its role is also reduced to that of a scaffold protein and an E3-ubiquitin ligase substrate. The coiled-coil domain of TRAF2, when engaged in a homotrimer complex, associates with the E3-ubiquitin ligase BIRC2. This in turn teams up with the E2-conjugating protein UBE2D3 (or others). Finally, BAG4 binds to non-occupied receptors and blocks inappropriate interaction with adaptors/effectors. Note the multiple ubiquitinylation sites on TRAF2 and RIPK1. (b) Molecular structure of the TNF receptor TNFRSF1A. The four cysteine-rich domains are indicated by green bars. CRD1 acts as a pre-ligand assembly domain (PLAD). CRD2 and CDR3 are involved in TNF binding. The intracellular segment possesses a death domain (DD). (c) Structure of the TRAF2 coiled-coil domain (CC) engaged in a homotrimer assembly and associated with the BIR1-domain of BIRC2.

that masks the nuclear localization signal (NLS). As a consequence, they remain in the cytosol and transcription activity is said to be inhibited. Phosphorylation of NFKBIA by IKBKB changes the situation because it leads to its destruction. The N-terminal phosphoserine-22 and -36 act as a ligand for the BTRC receptor subunit of the SCF E3-ubiquitin protein ligase complex. As a consequence, multiple ubiquitinylations follow on Lys-11 and -22. The K48-ubiquitinylated protein is now recognized by the proteasome and degraded. The dissociation of NFKBIA reveals the

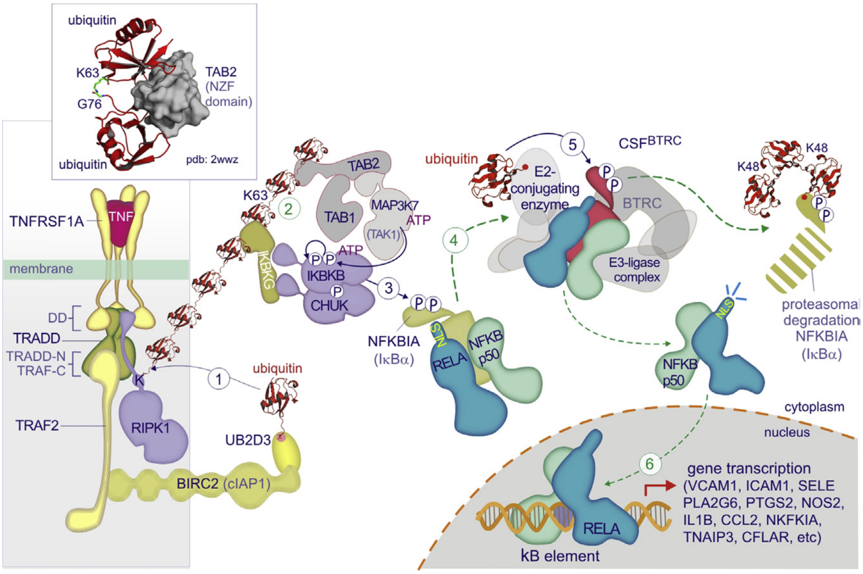


FIGURE 14-6 Signaling from the TNF receptor to the activation of NFKB. Recruitment of BIRC2 and its E2-partner UBE2D3 causes ubiquitinylation of RIPK1 (1). The resulting K63-ubiquitin chain acts as a docking site for the adaptor proteins IKBKG (NEMO) and TAB2 (2), each bringing along specific protein kinases. NEMO recruits CHUK (IKK-α) and IKBKB (IKK-β), whereas TAB2 associates with MAP3K7 (TAK1) together with its regulatory subunit TAB1. A series of phosphorylations follows, which leads to the activation of IKBKB. This then phosphorylates NFKBIA, the inhibitor protein of NFKB (also known as IκBα) (3). Phosphorylated NFKBIA serves as a ligand for the receptor BTRC, which is associated with the CSF ubiquitin ligase complex (4). This leads to ubiquitinylation of NFKBIA, this time in a K48-linked manner (5), a configuration that is recognized by the proteasome and subsequently degraded. This unmasks the nuclear localization signal of the NFKB transcription complex (comprising RELA and NFKBp50), which now migrates into the nucleus (6), to bind to its DNA-response element (κB). Numerous genes are transcribed (see also Figure 14-4).

NLS carried by NFKBp50, which directs the transcription factors to the nucleus (Figure 14-6).

NKFBp50 and RELA occupy the enhancer region of a large range of genes that control inflammation including the adhesion molecules ICAM1, selectins (SELE and SELP), and VCAM1, which are important for the recruitment of blood-borne cells into damaged/infected tissues (Verstrepen et al., 2008). As a result, TNF causes a transient upregulation of cell surface expression VCAM1 and ICAM1 and also of SELE and SELP on vascular endothelial cells. SELP, stored in specialized secretory granules, is almost instantly transferred to the plasma membrane, whereas expression of E-selectin, ICAM1, and VCAM1 occurs more slowly and then persists for a few days. The increased density of these adhesion molecules on the surface of endothelial cells enhances their “avidity” (the product, density × affinity) for leukocytes. In the second section of this chapter, the importance of increased avidity is revealed.

Web resource

For an extensive list of genes induced by TNF, we refer to the Kyoto Encyclopedia of Genes and Genomes (KEGG), pathway map 04668. URL: http://www.genome.jp/

Control of inflammation and apoptosis

The pathway triggered by TNF is self-limiting because NFKB also enhances the expression of NFKBIA (IκB), which initiates a negative feedback loop that attenuates the TNF signal. Among the negative feedback genes, we also find TNFAIP3 (formerly A20), an ubiquitin-editing enzyme particularly productive in the breakdown of K63-ubiquitin chains (return to Figure 14-4, gray-stained box with list of genes that "restrain" the inflammatory response (inhibitors)). These gene products secure the return to the basal expression of adhesion molecules of the vascular bed and thus ensure resolution of the inflammatory response. Another gene of importance is CFLAR, also known as FLIP, a regulator of apoptosis that inhibits assembly of the caspase-8 and FADD complex (hence its name caspase-8 and FADD-like apoptosis regulator). Enhanced expression of CFLAR prevents that accidentally formed signaling complexes between TNFRSF1A/TRADD/RIPK1/FADD lead to clustering and activation of the initiator caspases and thus of apoptosis.

TNF and cachexia Prolonged exposure to low concentrations of TNF causes the wasting syndrome, cachexia (from Greek, *kakos* bad, and *hexis* constitution). Indeed, cachectin, the mediator responsible for cachexia in mice exposed to trypanosome infection, has been identified as TNF (Beutler et al., 1985). Cancer patients suffer from cachexia, manifested as anorexia, early satiety, muscle wasting, weight loss, fatigue, and impaired immune response. Other inflammatory mediators that are involved in cachexia are interleukin-1 and -6 and INFγ. These are either secreted by the tumor alone or in concert with host-derived factors (Esper and Harb, 2005). They exert their effect directly on muscle metabolism, causing wasting of tissue, and indirectly by modifying neurotransmitter release in the hypothalamus (changing appetite, taste, and social behavior).

Linear ubiquitinylation is required to stabilize complex I

The above-mentioned sequence of events suffices to activate NFKB, but not for long and too weak, because an insufficient number of protein kinases are engaged and the complex is not fully stable. Failure to stabilize complex I may lead to TNF-mediated cell death. TNF trimers devoir of TRADD/TRAF2/BIRC2 may instead recruit the FADD/CASP complex (apoptosis) or it may cause the activation of RIPK1 which, after having teamed up with RIPK3, initiates the process of necroptosis (Holler et al., 2000). Although still very hard to grasp, both apoptosis and necroptosis may cause uncontrolled inflammation as made manifest by mice-lacking components that warrant a stable "complex I." They suffer from multiorgan inflammation, immune system deregulation, and dermatitis (Seymour et al., 2007).

Essentially, a productive NFKB pathway requires additional ubiquitin chains to enhance both the size and the stability of the assembled signaling complex (Haas et al., 2009; Gerlach et al., 2011). The additional ubiquitin chains are initiated by yet other E3-ubiquitin protein ligases. The first is a complex of proteins that give rise to linear ubiquitin chains. In these chains, the Gly-76 is connected with the initiation methionine (Met-1), giving rise to a true peptide bond. The E3-ligase complex therefore goes under the name of LUBAC, linear ubiquitin chain assembly complex. Its components are RNF31 (ring-finger protein 31, formerly HOIP), RBCK1 (RanBP-type C3HC4-type Zn-finger K?, formerly HOIL), and SHARPIN. RNF31 and RBCK1 are RING-finger-containing proteins and qualify as E3-ubiquitin protein ligases, but the essential component for stability of complex I is RNF31. It binds K63-ubiquitin chains with a Zn-finger motif (NZF1) and, with the help of an E2 ubiquitin-conjugating enzyme such as UBE2L3, it causes linear ubiquitinylation of diverse proteins such as RIPK1, TRADD, TRAF2, or IGBKG (NEM0) (Figure 14-7) (Stieglitz et al., 2013). These linear ubiquitin chains in turn recruit more IKBKG/IKBKA/IKBKB complexes, thus enhancing the signal (Rahighi et al., 2009). Moreover, linear chains are less sensitive to the action of CYLC (ubiquitin-editing complex that breaks down K63-chains), and this too may contribute to signal strength. The multitude of ubiquitin chains may also enhance the signal by offering additional binding sites, such as that occurs in BIRC2, which binds TRAF2 and also harbors an UBA (ubiquitin-associated) domain which interacts with ubiquitin (independent of the linkage). Proteins attached to each other and through linear ubiquitin chains may find it harder to dissociate from complex I (Kozlov et al., 2007).

Control of inflammation by apoptotic neutrophils and T cells. The final resolution of the inflammatory response not only requires that transcription of inflammatory mediators ceases: they must also be eliminated

continued

from the tissues. Massive apoptosis of neutrophils and T cells is a hallmark in the termination of inflammation. While dying, and before being eliminated by macrophages or other tissue cells, these leukocytes express high levels of the chemokine receptor CCR5. This also acts as a sink that sequesters the chemokines CCL3, -4, and -5. Mice lacking CCR5 manifest a prolonged presence of inflammatory mediators in their tissues (Ariel et al., 2006). Apoptosis is not a general mode to prevent abusive inflammation. Quite in contrast to the above-described phenomenon, loss of RIPK1 in intestinal or epidermal epithelial cells in mice leads to excessive inflammation manifested by the symptoms of (lethal) colitis and psoriasis, respectively. The reason behind the pathologies is excessive cell death of epithelial cells (Dannappel et al., 2014; Takahashi et al., 2014). What determines whether or not the cell engages the apoptosis/necroptosis pathway is not clear to us. Failure to occupy the trimerized receptors with complex I may automatically lead to enhanced recruitment of FADD, CASP8/10, or, alternatively, facilitate the onset of the necroptosis process (return to Figure 14-3 for the role of RIPK1 in TNF signaling).

A last E3-ubiquitin protein ligase to be mentioned is Pellino-3, initially discovered in *Drosophila melanogaster* as a protein interaction partner of Pelle (ortholog of IRAK4) (return to Chapter 13, “Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation,” Figure 13-2). In flies, it is required for innate immunity against Gram-positive bacteria (Grosshans et al., 1999; Moynagh, 2014). In the context of TNF signaling, it binds to RIPK1 and causes its ubiquitinylation (K63- or K11-ubiquitin chain), thus preventing the association of FADD/CASP8 complexes and inhibiting apoptosis (Yang et al., 2013).

Web resource

You can search ubiquitinylation sites in proteins in the protein modification resource PhosphoSitePlus. Type for instance the protein name “RIPK1” and, once on the protein page, scroll down to “modification sites and domains.” Note that certain lysines are not ubiquitinylated but acetylated instead (e.g., K530, K642, and K648).

Signaling via the MAP-kinases p38 and JNK

Complex I also serves as a platform for the activation of a number of MAP kinases. The sequence starts with MAP3K7, attached to TAB1, which activates MAP2K3 (MEK3) and MAP2K4 (MEK4). These in

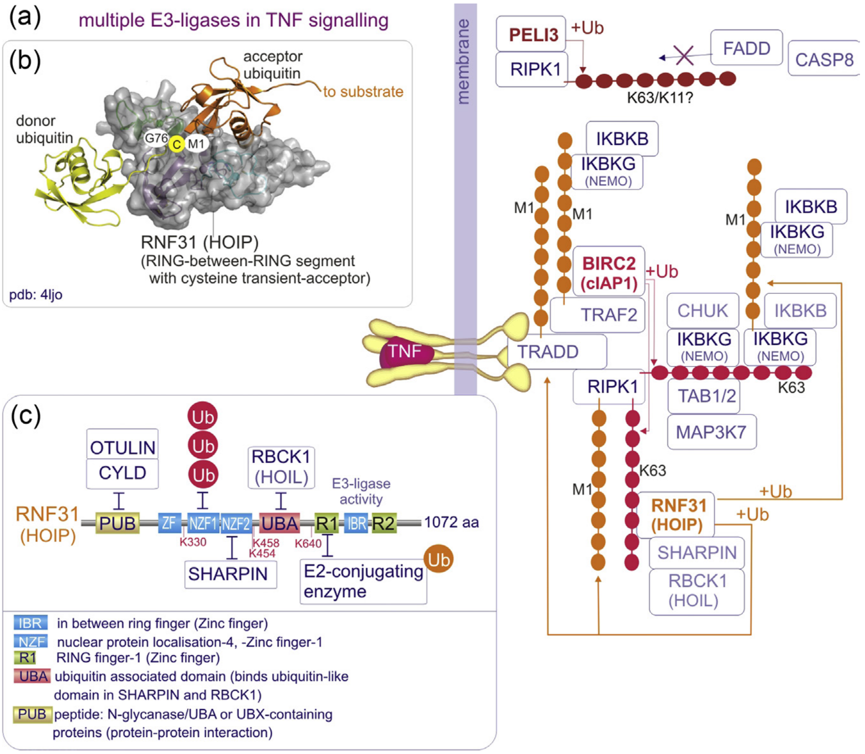


FIGURE 14-7 Multiple E3-ligases. (a) BIRC2 (cIAP2) plays an essential role in initiating the formation of complex I, but other ligases contribute to strengthen the signal and to avoid apoptosis or necroptosis. This occurs through ubiquitin-mediated stabilization and additional recruitment of IKBKG (NEMO)/IKBKB (IKK-β) complexes. Recruitment of RNF31, associated with SHARPIN and/or RBCK1, brings about a set of additional ubiquitin chains on proteins such as RIPK1, TRADD, and IKBKG. These are linear chains (methionine-1 linked to glycine-76) and are recognized by IKBKG (NEMO), thus recruiting more kinases into the complex. Since a number of proteins carry ubiquitin-binding motifs, for instance the UBA motif of BIRC2, the new chains may also serve to reinforce protein–protein interaction. (b) The RING-between-RING segment of RNF31 binds two ubiquitins, one covalently attached to the transient acceptor site (cysteine) and the other linked to substrate. The relative position of the two ubiquitins favors an M1–G76 (linear) linkage. Image courtesy of Dr Katrin Rittinger, National institute for medical research, London, UK. (c) Domain architecture of RNF31. Note the multiple protein interaction motifs, which allow binding to ubiquitin chains, to the partners RBCK1 and SHARPIN, and to the negative regulators CYLD (ubiquitin-editing enzyme that breaks K63 bonds) and OTULIN (ubiquitin-editing enzyme that breaks M1-bonds). The R1–IBR–R2 domains constitute the active site (E-ubiquitin protein ligase).

turn phosphorylate and activate MAPK14 (p38α) and MAPK8 (JNK1). Members of the AP1 family of transcription factors ATF2 and JUN are substrates of JNK, and their phosphorylation contributes to the expression of TNF-induced genes. JUN/ATF2 bind enhancer elements positioned near the κB-enhancer element and they act in a cooperative manner with NFKBp50/RELA (see Figure 14-4). Return to Chapter 13, “Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation," Figure 13-10, for more information about the whereabouts of these protein kinases. TRAF2 also acts as a scaffold for MAP3K1 (MEKK), which may play a role in the activation of CHUK and IKBKB and also phosphorylates and activates MAP2K4 (MEK4).

CHEMOKINES AND ACTIVATION OF INTEGRINS ON LEUKOCYTES

A family of chemokines

The action of TNF has caused the upregulation of adhesion molecules and the liberation of chemokines by the endothelial cells. Here we show how both play a role in the process where blood-borne cells leave the circulation (transendothelial migration or diapedesis) and penetrate the wounded/infected tissue. Although they are technically cytokines, the chemokines form a family of small structurally related proteins (8–14 kDa), originally identified and characterized as chemotactic agents. Leukocytes, when attached to substrate, are capable of sensing concentration differences of chemokines as small as 1% over a distance of 40 μm, approximating the linear dimensions of a cell. Under in vitro conditions, they respond by moving in the direction of the source in the process called chemotaxis (Zigmond, 1977).

With respect to transendothelial migration, it is not chemotaxis that directs the movement because the cells are floating free and buffeted by the blood. In this context, chemokines "attract" leukocytes by stimulating their attachment to and subsequent migration across the endothelial barrier. It is believed that the chemokines, generated by inflamed tissues, are presented on to the surface of the endothelial cell layer attached to long glycosaminoglycan chains (proteoglycans). This has been demonstrated for CXCL8 (IL-8) (Middleton et al., 1997; Proudfoot, 2006). What this means is that circulating leukocytes have first to attach to endothelial cells to be properly exposed. The detection of the chemokines activates adhesion molecules, members of the β1- and β2-integrins on the leukocytes, initiating a series of events that leads to their arrest, flattening, and transendothelial migration (Figure 14-14). By attracting leukocytes to sites of infection/inflammation, chemokines mediate an essential first step in host defense, tissue repair, and healing.

Chemokines became a focus of attention when it was shown that the envelope glycoprotein of human immunodeficiency virus (HIV) competes for binding with chemokines. Altogether with CD4, the chemokine receptors are the part of entry of HIV-1 into T-lymphocytes. Occupation of chemokine receptors, for instance of CXCR4, prevents the penetration

continued

of cells by the HIV-1 (Oberlin et al., 1996; Bleul et al., 1996). In some rare individuals, due to a homozygous mutation, the cytokine receptor CCR5 is not exposed at the cell surface. This receptor too acts as a coreceptor for HIV, and the virus cannot gain entry. These fortunate people appear to be immune to infection by HIV-1 (Liu et al., 1996). The mutation does not appear to induce any deleterious phenotype, which indicates that there is some redundancy among these receptors. Essential functions are well covered in the absence of CCR5.

About 40 human chemokines have been identified. They mainly act on neutrophils, monocytes, lymphocytes, eosinophils, endothelial cells, and their (stem cell) precursors (Lapidot et al., 2005; Dar et al., 2005). The chemokines are small proteins of roughly 100 amino acids having conserved cysteines that form essential intramolecular disulfide bonds. CX3CL1 (fractaline) makes an exception, it is composed of 397 amino acids, carries a number of glycan branches, and occurs as a transmembrane or cleaved soluble protein. Chemokines are classified as C, CC, CXC, and CX3C chemokines, according to the number of cysteines and the spacing between the first two cysteines in the N-terminal segment (Figure 14-8) (Zlotnik and Yoshie, 2000).

Chemokines bind G-protein-coupled receptors

Of the 19 human receptors that have been characterized (plus one or two splice variants), most recognize more than one chemokine. They are named after the class of chemokine they recognize: XCR, CCR, CXCR, or CX3CR. They are linked to both pertussis toxin-sensitive (Gαi/Gαo) and -insensitive G-proteins (Gα12/Gα13). For those acting on Gαi, it is predominantly the Gβγ subunits that carry the signal into cell (Arai et al., 1997; Neptune and Bourne, 1997), but in other cases, such as for the bacterial chemokine fMLP, activation also occurs through Gα12 and Gα13 (Van Keymeulen et al., 2006). The chemokines elicit a number of signal transduction events having different biological effects, which are illustrated in Figure 14-9 (Baggiolini, 1998).

Web resource

A good overview of chemokines and their receptors is provided by Wikipedia, URL: http://en.wikipedia.org/wiki/Chemokine.

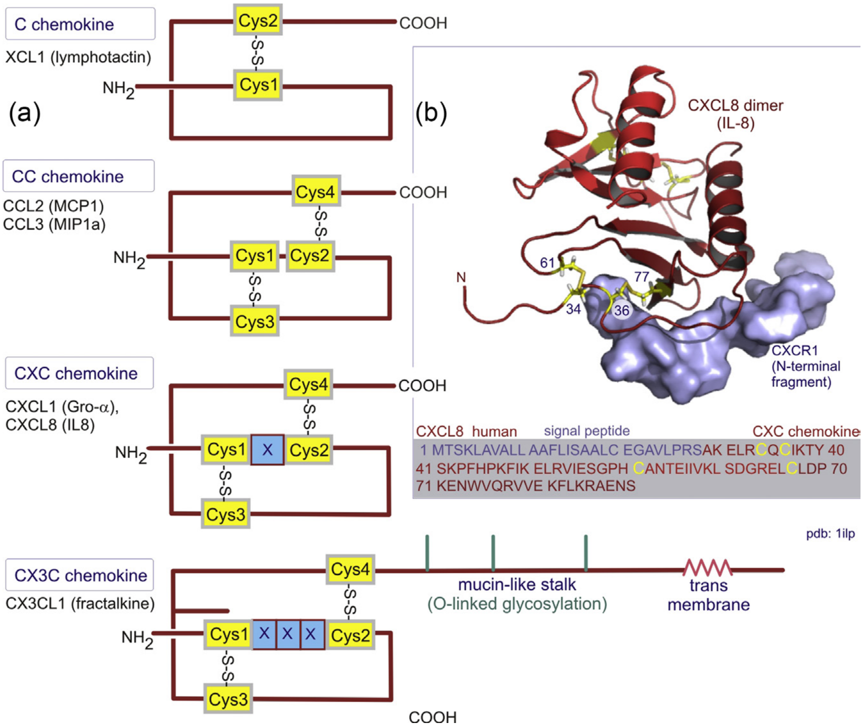
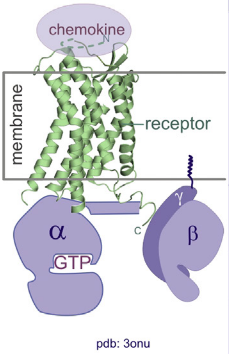


FIGURE 14-8 Chemokine structure. (a) Schematic representation of intramolecular disulphide bonding in C, CC, CXC, and CX3C chemokines. Only one or two examples are shown. CC and CXC each comprise some 20 members, the other two count only one or two. (b) Molecular structure of a CXCL8 dimer bound to the N-terminal fragment of the CXCR1 receptor. The following sequence indicates the position of the cysteines.



enzyme	products	biological effect	
phospholipase A2 (PLA2G6)	-lysophospholipid -free fatty acid (arachidonate)	leukotrienes ↑ lipoxygenase (ALOX5) ↑ arachidonate ↓ cyclo-oxygenase (PTGS1) ↓ prostaglandins	"eicosanoids" LTB4 - chemotactic agent PGE2 - relax vasc smooth muscle (redness) - permeability endothelium (swelling) - pain
phospholipase C (PLCB2, PLCG1)	-IP$_3$ -diacylglycerol	-elevation of intracellular free Ca^{2+} - exocytosis -activation of integrins via RASGRP2/RAP1	
phospholipase D (PLD1)	-phosphatidic acid -choline	-activation of the respiratory burst (NADPH oxidase or PHOX)	
PI 3-kinase-γ (PIK3CG)	-PI-3,4,5-phosphate -PI-3,4-phosphate	-regulation of actin cytoskeleton-cell spreading and migration	

FIGURE 14-9 Signals emanating from chemokine receptors and some of their biological effects in leukocytes. Chemokine receptors activate PI3-kinase and a number of phospholipases. The products of phospholipase A2 are released as messengers that play an important role in the manifestation of inflammation (redness, swelling, and pain). Phospholipase Cβ induces the production of IP$_3$ and diacylglycerol, which, among many possible actions, cause activation of integrins and liberation of granules. Phospholipase D produces phosphatidic acid, which activates the respiratory burst (production of reactive oxygen species). Finally, PI3-kinase produces 3-inositol phosphorylated phosphatidylinositol lipids, which play a role in cell spreading and cell migration.

Activation of integrins

Although the activation of the β1- and β2-integrins can be elicited by overexpression of different protein kinase C (PRKC) isoforms or by phorbol esters (Wright and Silverstein, 1982; Hogg et al., 2003), there is the problem that chemokine-induced integrin activation is not prevented when PRKC is inhibited (Laudanna et al., 1996). The stimulatory effect of phorbol ester can also be explained through its direct effect on the exchange factor RASGRP2 (guanyl-releasing protein or guanine-nucleotide exchange factor, formerly CalDAG-GEF1) causing activation of the GTPase RAP1B (Crittenden et al., 2004) (Figure 14-10 and Figure 14-11 for domain architecture). The natural source of activation is of course diacylglycerol, produced by phospholipase-β2 (PLCB2). Rap1B (Pizon et al., 1988; Kitayama et al., 1989; Zwartkruis and Bos, 2000), gained further physiological significance with the realization that it activates integrins

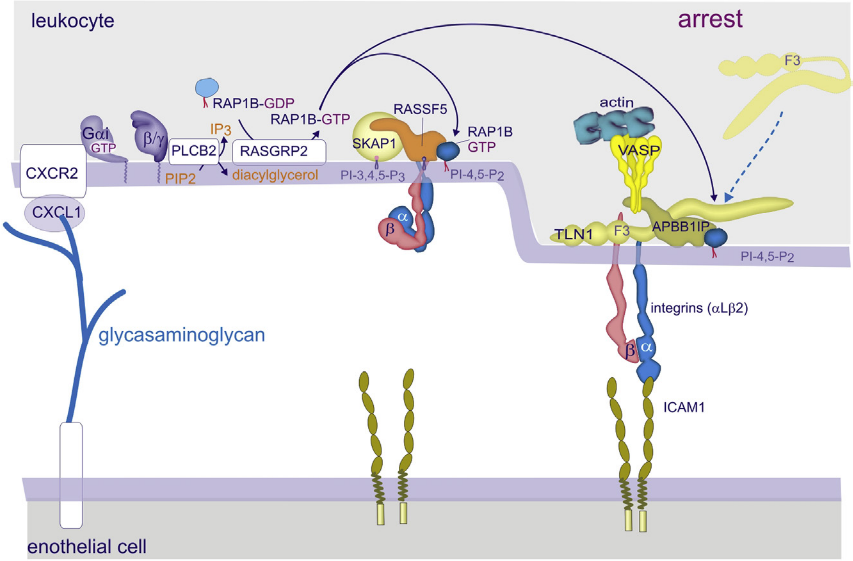


FIGURE 14-10 Chemokine-mediated activation of integrins in leukocytes. Binding of the chemokines, example of CXCL1 (Gro-α) attached to a glycosaminoglycan chain, causes activation of Gαi (GNAI1). The liberated Gβγ subunits activate PLCβ2 (PLCB2), giving rise to diacylglycerol and IP_3, both of which are involved in the activation of RASGRP2, a guanine-nucleotide exchange factor, which activates RAP1. This binds its effectors RASSF5 (RapL) and APBB1IP (Riam) responsible for the activation of integrins (e.g., αLβ2 (LFA1)). PI-4,5-P2 and PI-3,4,5-P3 facilitate membrane recruitment of SKAP1, which brings RASSF5 to the membrane, and of TLN1 (Talin), an essential component in the unfolding activation process of the integrin outer segment. APBB1IP also interacts with VASP, and this opens the possibility to elaborate an actin cytoskeleton so to enforce the adhesion contact.

in a number of blood-borne cells, including platelets (Franke et al., 1997; Reedquist et al., 2000; Liu et al., 2002; Shimonaka et al., 2003). Activated RAPB1 interacts with RASSF5 (formerly RapL) and APBB1IP (formerly Riam) (Lafuente et al., 2004; Katagiri et al., 2003) and this causes both activation and clustering of integrins, thereby augmenting their affinity and avidity for ligands (Han et al., 2006). APBB1IP interacts with the cytoskeletal protein TLN1 (Talin), and this in turn binds integrins and thus modifies their configuration (Campbell and Ginsberg, 2004). APBB1IP also recruits VASP, leading to the organization of the actin cytoskeleton so to enforce the adhesion contacts (Ridley et al., 2003; Nourshargh et al., 2010). Epithelial cells seem to employ a similar mode of action, albeit it with different RAP1 nucleotide exchange factors (return to Chapter 11, “Signal Transduction to and from Adhesion Molecules” (Figures 11-14 and 11-15). The activation mechanism is detailed in Chapter 11, “Signal Transduction to and from Adhesion Molecules” (Figures 11-8 and 11-14(b)). Integrin activation causes arrest of the leukocytes on endothelial cells that possess the ligands VCAM1 and ICAM1, both of which are highly expressed due to the presence of TNF (and other inflammatory cytokines). In this way, floating leukocytes take on the characteristics of adherent cells (Figure 14-14).

RASGRP2, also known as CalDAG-GEF1, is a Ca^{2+} and diacylglycerol-activated guanine nucleotide exchange factor. It contains a C1 domain, first identified as a conserved region in the members of the PRKC family, through which it binds diacylglycerol. It is worth noting that there are numerous proteins containing C1 domains, and so they are potential targets of the action of diacylglycerol or phorbol esters (return to Chapter 9, “Protein Kinase C in Oncogenic Transformation and Cell Polarity,” Figures 9-1 and 9-2). It also contains an EF-motif, which confers Ca^{2+} sensitivity (dealt with in Chapter 6, “Intracellular Calcium,” Figure 6-03.

Depending on the type of leukocyte, different integrins participate in the firm arrest to endothelium. For instance, in monocytes, αMβ2 (MAC1) is predominantly involved in arrest, whereas in neutrophils this role is taken by αLβ2 (LFA1). Lymphocytes rely mainly on α4β1 (VLA4) (return to Chapter 11, “Signal Transduction to and from Adhesion Molecules,” Figures 11-3, 11-6, and 11-7).

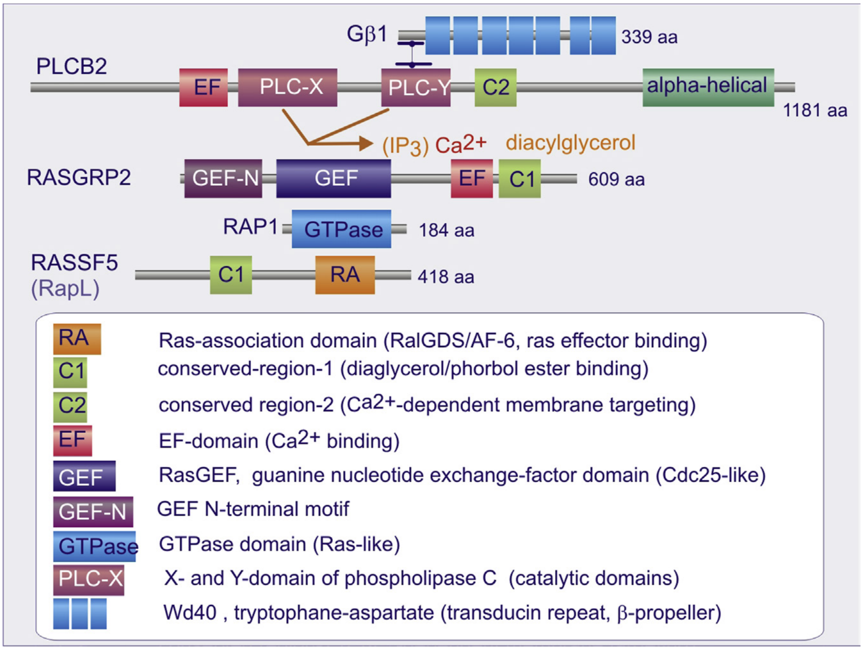

Gβ1
339 aa
PLCB2
EF
PLC-X
PLC-Y
C2
alpha-helical
1181 aa
(IP3) Ca2+
diacylglycerol
RASGRP2
GEF-N
GEF
EF
C1
609 aa
RAP1
GTPase
184 aa
RASSF5
(RapL)
C1
RA
418 aa
RA
Ras-association domain (RalGDS/AF-6, ras effector binding)
C1
conserved-region-1 (diaglycerol/phorbol ester binding)
C2
conserved region-2 (Ca2+-dependent membrane targeting)
EF
EF-domain (Ca2+ binding)
GEF
RasGEF, guanine nucleotide exchange-factor domain (Cdc25-like)
GEF-N
GEF N-terminal motif
GTPase
GTPase domain (Ras-like)
PLC-X
X- and Y-domain of phospholipase C (catalytic domains)
Wd40 , tryptophane-aspartate (transducin repeat, β-propeller)

FIGURE 14-11 Domain architecture of components of the integrin activation pathway in leukocytes. The N-terminal segment of the Gβ1 subunit binds PLCB2 leading to the production of diacylglycerol and IP_3. IP_3 liberates Ca^{2+} from intracellular stores. Altogether, Ca^{2+} and diacylglycerol recruit the guanine-nucleotide exchange factor RASGRP2 to the membrane and cause its activation, leading to GTP loading of RAP1. RAP1-GTP binds to the RAS-association domain (RA) of RASSF5 (RapL) and this plays a role in the activation process of integrins.

CELLULAR PROTRUSIONS AID IN PROBING PERMISSIVE SITES ON THE ENDOTHELIAL SURFACE

The leukocytes flatten and crawl on the endothelial surface in search for endothelial cellular junctions. This involves important reorganization of the cytoskeleton in which the Gβγ subunits, derived from Gαi, again play an essential role. They activate the p110γ subunit of class-I PI3-kinase (PIK3CG) inducing the generation of phosphatidyl-3,4,5-phosphate (subject discussed in Chapter 16, “Signaling through the Insulin Receptor”). This engages guanine-nucleotide exchange factors that activate both RAC1 and CDC42 (members of the Rho family of GTPases) (Fleming et al., 2004) (Figure 14-12 and 14-13 for domain architecture). In particular, RAC1 is a key in the initiation of a branched network of actin filaments and in orchestrating the formation of lamellipodia (protrusions), which cause cell spreading (Pollard and Borisy, 2003). RAC1 is activated by a number of GEFs of which two, TIAM1 and DOCK1, qualify in the context of leukocyte

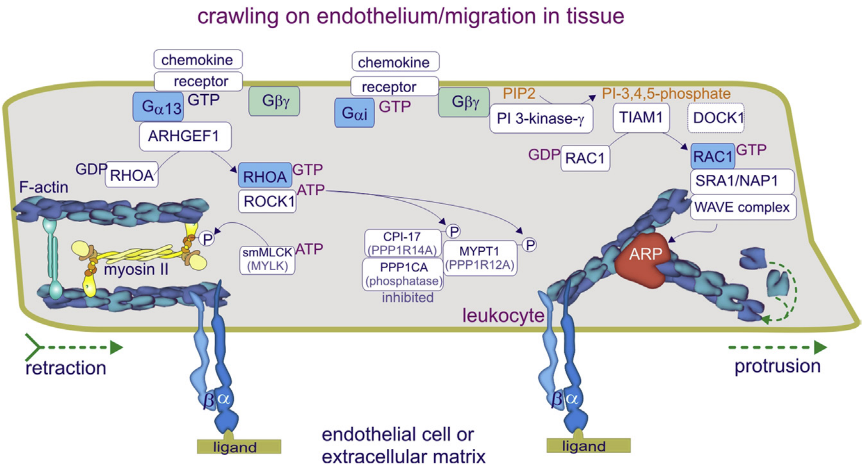


FIGURE 14-12 Members of the Rho family of GTPases are instrumental in the control of cell migration. Chemokine receptors are linked to heterotrimeric GTP-binding proteins. With respect to the formation of protrusions (at the leading edge), it is the Gβγ-subunits that are of importance. These bind PI3-kinase-γ resulting in the formation of PI-3,4,5-phosphate. This attracts and activates the RAC1-guanine nucleotide exchange factor TIAM1. GTP-loaded RAC1 binds SRA1/NAP1, a component of the WAVE2 complex, and this in turn interacts with ARP2/3. It is bound to the existing actin filaments and catalyzes the nucleation of new branches. The elongating actin filaments push the membrane forward. With respect to retraction at the rear of the cell, chemokine receptors mediate their effect through Gα13 which binds ARHGEF1. GTP-loaded RHOA interacts with and activates the serine/threonine kinase ROCK1. This causes the phosphorylation of regulatory subunits (CPI-17 and MYPT) that inhibit the serine/threonine phosphatase PPP1CA. A loss of phosphatase leads to an enhanced phosphorylation of myosin-light chain (by smMLCK), which enables ATP hydrolysis at the head of myosin-II, resulting in movement and sliding of the actin filaments (contracting stress fibers).

migration. Activated RAC1 binds to SRA1/NAP1, two large proteins that interact with WAVE2 that is enabled to bind to ARP2/3 (return to Chapter 11, “Signal Transduction to and from Adhesion Molecules,” Figure 11-20) (Miki et al., 1998; Suetsugu et al., 2006). This complex of actin-like proteins binds to the existing actin filaments and catalyzes the further polymerization of actin, thus initiating new filaments that press the membrane forward. In Chapter 6, “Intracellular Calcium,” Section “Ca^{2+} in action,” we have shown that besides the formation of a RAC1-driven formation of an actin meshwork, contractile actin filaments also play a role in pulling the dorsal membrane down, thereby, most likely, generating hydrostatic pressure that aids the outward movement of the membrane (see Figure 6-22).

Crossing the endothelium

Directionality is not necessary for leukocyte binding to endothelial cells and for transendothelial cell migration. What matters is that the activated integrins find the intercellular junctions. While crawling, ventral protrusions probe the endothelial surface for "permissive sites." The process of transendothelial migration is initiated by binding of integrins, initially involved in the arrest of leukocytes, to adhesion molecules that constitute the tight junctions. Thus, integrin αLβ2 (LFA1) shifts its attention from ICAM1 toward F11R (JAM1) (Martin-Padura et al., 1998; Del Maschio et al., 1999). α4β1 (VLA4) shifts its attention from VCAM1 toward JAM2 (Cunningham et al., 2002), and αMβ2 (Mac-1) turns from ICAM1 toward JAM3 (Arrate et al., 2001; Santoso et al., 2002). The binding of integrins occurs at the membrane proximal to the immunoglobulin domain of the JAMs, and it is believed that this serves to guide the leukocyte across the paracellular space. For the composition of the tight junctions, return to Chapter 11, "Signaling to and from Adhesion Molecules," Figure 11-5).

Additional support for transendothelial migration comes from the ICAM clusters that have developed through the interaction with clusters of integrins on the leukocytes. In an as yet undescribed mechanism, these ICAM clusters activate the tyrosine protein kinases SRC and PTK2B (formerly PYK2). These in turn phosphorylate VE-cadherin (CDH5), a component of the adherens junction. This leads to the loss of the interaction with β-catenin (CTNNB1), an event that destabilizes the cell–cell junction. Disengaged VE-cadherins are removed from the lateral membranes, and the path is cleared for the passing leukocyte. In parallel with the uptake of VE-cadherin, the tyrosine phosphatase PTPRB is also removed from the surface, thereby facilitating the work of SRC and PTK2B. Return to Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation," in particular Figure 12-16, for a reminder of the role of phosphorylation in cadherin-mediated adhesion.

OTHER ROLES OF CHEMOKINES

Dendritic cells also generate chemokines, in particular CCL5 (RANTES), CCL10, CCL17, CCL18, CCL19, and CCL25. This suggests a role for chemokines in the initiation of immune responses through possible adjuvant properties. It may enable dendritic cells to attract T-cell subsets. Some chemokines are constitutively expressed and may have a homeostatic rather than inflammatory role. Mice that lack CXCL12 (SDF-1) die perinatally and show a defect in the generation of B-cells resulting from a disorganized bone marrow stromal environment (Nagasawa et al., 1996). Failure to express CCL21 in lymph nodes causes depletion of T cells, possibly due to a failure of their homing capacity (Gunn et al., 1999).

Paracellular or transcellular migration? Leukocytes cross the endothelium in two modes: paracellular (in between cells as described here) or transcellular (through the cell). The latter involves the process of transcytosis, where leukocytes cross the cell within a caveolin-coated transport vesicle. It is generally agreed that both mechanisms are employed under flow conditions. However, under static conditions, leukocytes cross by means of transcellular migration only (Nourshargh et al., 2010).

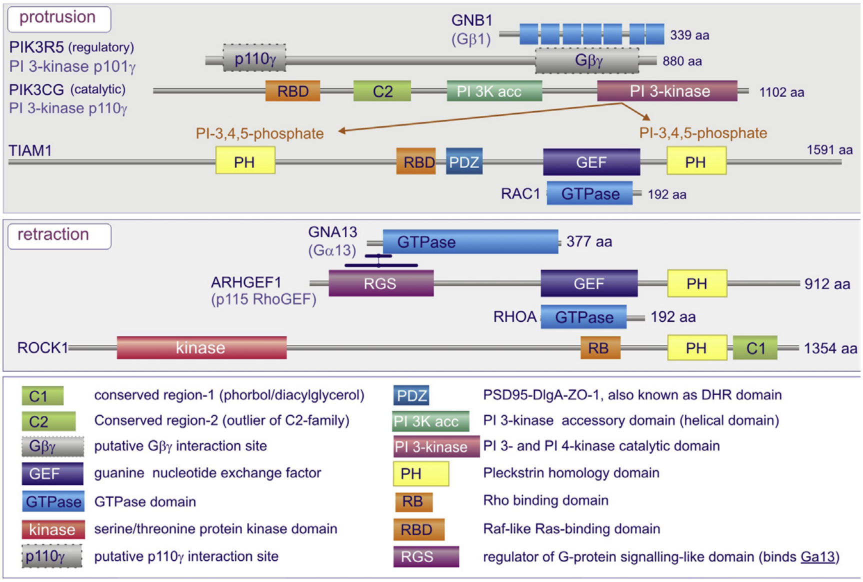

protrusion
GNB1
(Gβ1)
339 aa
PIK3R5 (regulatory)
PI 3-kinase p101γ
p110γ
Gβγ
880 aa
PIK3CG (catalytic)
PI 3-kinase p110γ
RBD
C2
PI 3K acc
PI 3-kinase
1102 aa
PI-3,4,5-phosphate
PI-3,4,5-phosphate
TIAM1
PH
RBD
PDZ
GEF
PH
1591 aa
RAC1
GTPase
192 aa
retraction
GNA13
(Gα13)
GTPase
377 aa
ARHGEF1
(p115 RhoGEF)
RGS
GEF
PH
912 aa
RHOA
GTPase
192 aa
ROCK1
kinase
RB
PH
C1
1354 aa
C1 conserved region-1 (phorbol/diacylglycerol)
C2 Conserved region-2 (outlier of C2-family)
Gβγ putative Gβγ interaction site
GEF guanine nucleotide exchange factor
GTPase GTPase domain
kinase serine/threonine protein kinase domain
p110γ putative p110γ interaction site
PDZ PSD95-DlgA-ZO-1, also known as DHR domain
PI 3K acc PI 3-kinase accessory domain (helical domain)
PI 3-kinase PI 3- and PI 4-kinase catalytic domain
PH Pleckstrin homology domain
RB Rho binding domain
RBD Raf-like Ras-binding domain
RGS regulator of G-protein signalling-like domain (binds Ga13)

FIGURE 14-13 Domain architecture of proteins involved in signaling leukocyte migration. Protrusion: Gβγ subunits (dissociated from Gαi) interact with PIK3R5 (p101γ) causing activation of the catalytic subunit of PI3-kinase PIK3CG (p110γ). TIAM1 binds to PI-3,4,5-phosphate through its PH domains. RAC1 interacts with the GEF domain and this leads to guanine nucleotide exchange. Retraction: The RGS domain of ARHGEF1 (p115Rho-GEF) binds to the N-terminal of GNA13 ($G\alpha_{13}$). As a consequence, RHOA is loaded with GTP and interacts the Rho-binding domain of its effector ROCK1. This activates the protein kinase.

MIGRATION WITHIN THE TISSUE

Having traversed the endothelial intercellular junction and then the basal membrane, leukocytes search for sites of infection, migrating to the gradient of chemokines (such as formylmethionyl peptides) released by bacteria. This movement is characterized by the assembly of actin fibers into protrusions at the leading edge of the cell and by formation of contractile actin–myosin complexes at the rear and along the sides. Both these processes are initiated following receptor activation of the heterotrimeric G-proteins, Gαi controlling forwardness and Gα12/13 controlling backness (Mayadas and Cullere, 2005). As already related, Gβγ subunits derived from Gαi induce formation of PI-3,4,5-trisphosphate. This occurs at the point of the cell closest to the source of the chemokine, in this way positioning the leading edge (Figure 14-12). The GTPase RAC1 focuses membrane protrusions uniquely at the leading edge while the role of CDC42 is to ensure that there is no formation of secondary protrusions elsewhere (Van Keymeulen et al., 2006). Retraction of the cell body at the rear is mediated through the activation of the α-subunits of Gα12 or Gα13 (Alblas et al., 2001). This occurs through the formation of actin stress fibers formed by antiparallel filaments of actin intercalated with myosin II. Activation of the myosin II occurs through phosphorylation of the myosin light chain (MLC) in a pathway involving ARHGEF1 RHOA and ROCK1. For more detail, return to Chapter 6, “Intracellular Calcium,” Figure 6-23.

In the tissue, the activated integrins augment neutrophil responses such as phagocytosis, exocytosis, and generation of reactive oxygen metabolites, necessary for the killing and clearing of microorganisms. They also determine the life span of the leukocyte in the tissues (Mayadas and Cullere, 2005).

Cellular protrusions come under different names: podosomes, invadopodes, lamellipodia, or, in the case of neutrophils, pseudopods. They vary with respect to molecular composition and shape.

Web resource

For a full overview of signaling events involved in leukocyte arrest and migration, consult the Kyoto Encyclopedia of Genes and Genomes, KEGG Pathway map 04670 “leukocyte transendothelial migration.” URL: www.genome.jp.

Role of vascular pericytes

Freshly emigrated neutrophils and monocytes can acquire both pro-migratory and pro-survival signals from pericytes (attached to arterioles/capillaries, Figure 14-2). The contact between the cells provides novel instructions by which the leukocytes migrate more efficiently toward the injured or infected foci. The chemokine MIF (macrophage migration inhibitory factor) produced by the pericytes causes enhanced cell surface expression of toll-like receptors (sensing nonself), as well as several pro-adhesive and pro-migratory molecules, including the receptor for MIF (CD74), MMP9, MMP14, β1-integrins, and G-protein-coupled receptors for formyl peptides (Stark et al., 2013; Alon and Nourshargh, 2013). Return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptors and Signaling through Ubiquitinylation" for more information about toll-like receptors and their role in innate immunity.

THE THREE-STEP PROCESS OF LEUKOCYTE ADHESION TO ENDOTHELIAL CELLS

All these processes form part of a more general sequence of events that is now referred to as the three-step process of leukocyte adhesion to endothelial cells. Leukocytes make contact with the vascular endothelium from time to time by the presentation of selectin surface ligands or receptors (SELPLG, PODXL2, or SELL see above) that bind to ligand or receptors exposed on the endothelial cells (CD34, SELE, SELL) (see Chapter 11, "Signaling to and from Adhesion Molecules," Figure 11-12). These interactions tether the cells in a manner by which they can attach and then roll on the surfaces of the endothelial cells and finally detach (Figure 14-14). Increased expression of selectins (as induced by TNF) greatly facilitates the rolling process. The close contact with the endothelial surface allows leukocytes to verify the presence of chemokines attached on the surface by long glycosaminoglycan chains. Their integrins become activated so that they bind to ICAM1 or VCAM1, also present at elevated levels due to the presence of inflammatory mediators so causing their arrest. They flatten and become competent to pass through the endothelial layer (Butcher, 1991; Springer, 1994). Not all bound cells transmigrate. Some merely perch on the endothelial cell surface and then detach. Those that do succeed pass through the basal membrane and make it right through into the tissues.

Depending on the type of leukocyte, different integrins participate in their firm arrest to endothelium. For instance, in monocytes αMβ2 is predominantly involved in arrest, whereas in neutrophils this role is taken by αLβ2. Lymphocytes rely mainly on α4β1.

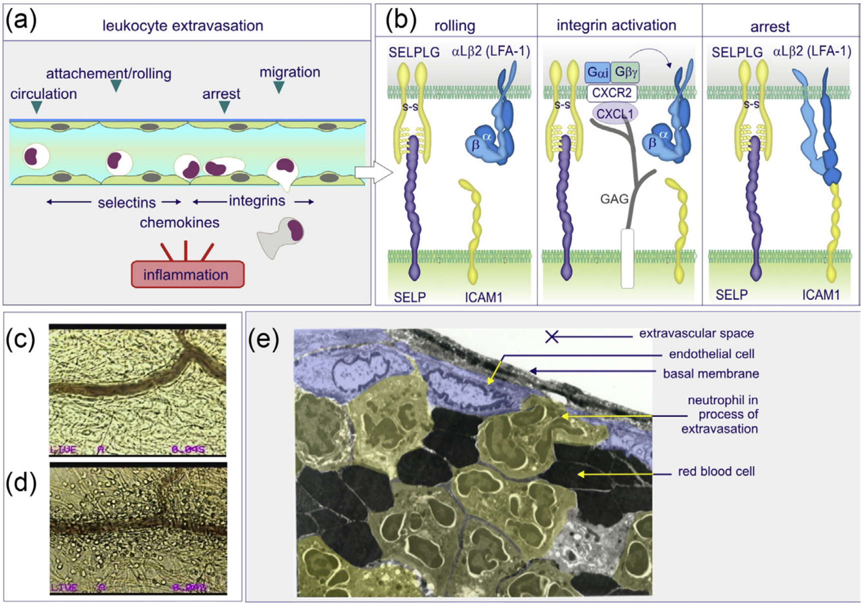


FIGURE 14-14 The three-step model of leukocyte transendothelial migration. (a) Representation of the three-step model of leukocyte transendothelial migration through the wall of a venule. This comprises (1) selectin-mediated rolling of leukocytes on the vascular endothelial cell surface, then (2) chemokine-mediated activation of integrins on the leukocytes resulting in (3) arrest and migration through the vascular endothelial cell layer. A large proportion of leukocytes is normally mildly attached and just roll along on the endothelial surface. Migration is triggered by firm arrest due to local inflammation as a consequence of the release of chemokines and enhanced expression of the selectins, VCAM1, and ICAMs. (b) A molecular representation of the same three steps, with gray (on top) representing the leukocyte and green (at the bottom) representing the endothelial cell. Left panel: Initial contact between the two cells is made through P-selectin (SELP) interacting with the SELPLG. This allows the cells to roll. Center panel: The close proximity to the endothelial cells allows detection of Gro-α (CXCL1, presented on a glycosaminoglycan chain) by its receptor CXCR2. This causes activation of GTP-binding proteins that in turn activate the integrin αLβ2 (LFA1). Right panel: The activated integrin now binds ICAM1 causing firm attachment of the leukocyte to the endothelial cell. This is a prerequisite for further flattening and then migration through the endothelial cell layer. (c) Video micrograph illustrating a rat mesenteric venule (~40 μm diameter). The blurring within the venule is due to the flow of blood. (d) Video micrograph illustrating leukocyte accumulation into the mesenteric tissue after upstream administration of the chemokine LTB4 (from Sussan Nourshargh, Imperial College, London). (e) Thin section electron micrograph, showing a leukocyte (yellow, multilobed nucleus) migrating through a layer of endothelial cells (blue) into rat mesenteric tissue. *From Sussan Nourshargh, Imperial College, London.*

References

Addison, W., 1843. Experimental and practical researches on the structure and function of blood corpuscles; on inflammation; and on the origin and nature of tubercles in the lungs. Trans. Prov. Med. Surg. Assoc. 11, 233–305.

Aggarwal, B.B., 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. 3, 745–756.

Alblas, J., Ulfman, L., Hordijk, P., Koenderman, L., 2001. Activation of Rhoa and ROCK are essential for detachment of migrating leukocytes. Mol. Biol. Cell 12, 2137–2145.

Alon, R., Nourshargh, S., 2013. Learning in motion: pericytes instruct migrating innate leukocytes. Nat. Immun. 14–15.

Anderson, B.F., Legallaies, F.Y., 1952. Vascular reactions of normal and malignant tissues in vivo: the role of hypotension in the action of a bacterial polysaccharide on tumors. J. Natl. Cancer Inst. 12, 1279–1295.

Arai, H., Tsou, C.L., Charo, I.F., 1997. Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: evidence that directed migration is mediated by betagamma dimers released by activation of Galphai-coupled receptors. Proc. Natl. Acad. Sci. U.S.A. 94, 14495–14499.

Ariel, A., Fredman, G., Sun, Y.P., et al., 2006. Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. Nat. Immunol. 7, 1209–1216.

Arrate, M.P., Rodriguez, J.M., Tran, T.M., Brock, T.A., Cunningham, S.A., 2001. Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. J. Biol. Chem. 276, 45826–45832.

Baggiolini, M., 1998. Chemokines and leukocyte traffic. Nature 392, 565–568.

Bennett, J.P., Hirth, K.P., Fuchs, E., Sarvas, M., Warren, G.B., 2006. The bacterial factors which stimulate neutrophils may be derived from procaryote signal peptides. FEBS Lett. 116, 57–61.

Beutler, B., Greenwald, D., Hulmes, J.D., Chang, M., Pan, Y.C., Mathison, J., Ulevitch, R., Cerami, A., 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature 316, 552–554.

Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., Springer, T.A., 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 382, 829–833.

Bruns, P., 1888. Die Heilwirkung des Erysipels auf Geschwülste (The healing effect of erysipelas on tumours). Beitr Klin. Chir. 3, 443–466.

Butcher, E.C., 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67, 1033–1036.

Campbell, I.D., Ginsberg, M.H., 2004. The talin-tail interaction places integrin activation on FERM ground. Trends Biochem. Sci. 29, 429–435.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U.S.A. 72, 3666–3670.

Cohnheim, J., 1882. Lectures on General Pathology. The New Sydenham Society, London.

Coley, W.B., 1891. Contribution to the knowledge of sarcoma. Ann. Surg. 14, 199–220.

Crittenden, R., Bergmeier, W., Zhang, Y., Piffath, C.L., Liang, Y., Wagner, D.D., Housman, D.E., Graybiel, A.M., 2004. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat. Med. 10, 982–986.

Cunningham, S.A., Rodriguez, J.M., Arrate, M.P., Tran, T.M., Brock, T.A., 2002. JAM2 interacts with alpha4beta1. Facilitation by JAM3. J. Biol. Chem. 277, 27589–27592.

Dannappel, M., Vlanntis, K., Kumari, S., Polykratis, A., Kim, C., Wachsmuth, L., et al., 2014. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature 513, 90–94.

Dar, A., Goichberg, P., Shinder, V., Kalinkovich, A., Kollet, O., Netzer, N., Margalit, R., Zsak, M., Nagler, A., Hardan, I., Resnick, I., Rot, A., Lapidot, T., 2005. Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. Nat. Immunol. 6, 1038–1046.

Del Maschio, A., De Luigi, A., Martin-Padura, I., Brockhaus, M., Bartfai, T., Fruscella, P., Adorini, L., Martino, G., Furlan, R., De Simoni, M.G., Dejana, E., 1999. Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is inhibited by an antibody to junctional adhesion molecule (JAM). J. Exp. Med. 190, 1351–1356.

Deng, G.M., Zheng, L., Chan, F.K., Lenardo, M., 2005. Amelioration of inflammatory arthritis by targeting the pre-ligand assembly domain of tumor necrosis factor receptors. Nat. Med. 11, 1066–1072.

Ea, C.K., Deng, L., Xia, Z.P., Pineda, G., Chen, Z.J., 2006. Activation of IKK by TNFalpha requires site-specific ubiquitinylation of RIP1 and polyubiquitin binding by NEMO. Mol. Cell 22, 245–257.

Esper, D.H., Harb, W.A., 2005. The cancer cachexia syndrome: a review of metabolic and clinical manifestations. Nutr. Clin. Pract. 20, 369–376.

Feldmann, M., Maini, R.N., 2001. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? Annu. Rev. Immunol. 19, 163–196.

Feldmann, M., Brennan, F.M., Williams, R.O., Cope, A.P., Gibbons, D.L., Katsikis, P.D., Maini, R.N., 1992. Evaluation of the role of cytokines in autoimmune disease: the importance of TNF alpha in rheumatoid arthritis. Prog. Growth Factor Res. 4, 247–255.

Fleming, I.N., Batty, I.H., Prescott, A.R., Gray, A., Kular, G.S., Stewart, H., Downes, C.P., 2004. Inositol phospholipids regulate the guanine-nucleotide-exchange factor Tiam1 by facilitating its binding to the plasma membrane and regulating GDP/GTP exchange on Rac1. Biochem. J. 382, 857–865.

Franke, B., Akkerman, J.W., Bos, J.L., 1997. Rapid Ca^{2+}-mediated activation of Rap1 in human platelets. EMBO J. 16, 252–259.

Gerlach, B., Cordier, S.M., Schmukle, A.C., Emmerich, C.H., Rieser, E., Haas, T.L., Webb, A.I., Rickard, J.A., Anderton, H., Wong, W.W., Nachbur, U., Gangoda, L., Warnken, U., Purcell, A.W., Silke, J., Walczak, H., 2011. Linear ubiquitinylation prevents inflammation and regulates immune signalling. Nature 471, 591–596.

Gomez, M.I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A., Prince, A., 2004. Staphylococcus aureus protein A induces airway epithelial inflammatory responses by activating TNFR1. Nat. Med. 10, 842–848.

Graille, M., Stura, E.A., Corper, A.L., Sutton, B.J., Taussig, M.J., Charbonnier, J.B., Silverman, G.J., 2000. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. Proc. Natl. Acad. Sci. U.S.A. 97, 5399–5404.

Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., Scheurich, P., 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 83, 793–802.

Grosshans, J., Schnorrer, F., Nüsslein-Volhard, C., 1999. Oligomerisation of Tube and Pelle leads to nuclear localisation of dorsal. Mech. Dev. 81, 127–138.

Gunn, M.D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L.T., Nakano, H., 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J. Exp. Med. 189, 451–460.

Haas, T.L., Emmerich, C.H., Gerlach, B., Schmukle, A.C., Cordier, S.M., Rieser, E., Feltham, R., Vince, J., Warnken, U., Wenger, T., Koschny, R., Komander, D., Silke, J., Walczak, H., 2009. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol. Cell. 36, 831–844.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J., Ginsberg, M.H., 2006. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr. Biol. 16, 1796–1806.

Hogg, N., Laschinger, M., Giles, K., McDowall, A., 2003. T-cell integrins: more than just sticking points. J. Cell Sci. 116, 4695–4705.

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.L., Schneider, P., Seed, B., Tschopp, J., 2000. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol. 1, 489–495.

Hsu, H., Xiong, J., Goeddel, D.V., 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81, 495–504.

Itoh, N., Nagata, S., 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. J. Biol. Chem. 268, 10932–10937.

Katagiri, K., Maeda, A., Shimonaka, M., Kinashi, T., 2003. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. Nat. Immunol. 4, 741–748.

Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., Noda, M., 1989. A ras-related gene with transformation suppressor activity. Cell 56, 77–84.

Kolb, W.P., Granger, G.A., 1968. Lymphocyte in vitro cytotoxicity: characterization of human lymphotoxin. Proc. Natl. Acad. Sci. U.S.A. 61, 1250–1255.

Kozlov, G., Nguyen, L., Lin, T., De Crescenzo, G., Park, M., Gehring, K., 2007. Structural basis of ubiquitin recognition by the ubiquitin-associated (UBA) domain of the ubiquitin ligase EDD. J. Biol. Chem. 282, 35787–35795.

Kristiansen, S.V., Pascual, V., Lipsky, P.E., 1994. Staphylococcal protein A induces biased production of Ig by VH3-expressing B lymphocytes. J. Immunol. 153, 2974–2982.

Kulathu, Y., Akutsu, M., Bremm, A., Hofmann, K., Komander, D., 2009. Two sided ubiquitin binding explains specificity of the TAB2 NZF domain. Nat. Struct. Mol. Biol. 166, 1328–1330.

Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B., Boussiotis, V.A., 2004. RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell 7, 585–595.

Lapidot, T., Dar, A., Kollet, O., 2005. How do stem cells find their way home? Blood 106, 1901–1910.

Laudanna, C., Campbell, J.J., Butcher, E.C., 1996. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. Science 271, 981–983.

Liu, R., Paxton, W.A., Choe, S., Ceradini, D., Martin, S.R., Horuk, R., MacDonald, M.E., Stuhlmann, H., Koup, R.A., Landau, N.R., 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 86, 367–377.

Liu, L., Schwartz, B.R., Tupper, J., Lin, N., Winn, R.K., Harlan, J.M., 2002. The GTPase Rap1 regulates phorbol 12-myristate 13-acetate-stimulated but not ligand-induced beta 1 integrin-dependent leukocyte adhesion. J. Biol. Chem. 277, 40893–40900.

Marchesi, V.T., Florey, H.W., 1960. Electron micrographic observations on the emigration of leucocytes. Quart. J. Exp. Physiol. 45, 343–348.

Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., Dejana, E., 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J. Cell Biol. 142, 117–127.

Mayadas, T.N., Cullere, X., 2005. Neutrophil beta2 integrins: moderators of life or death decisions. Trends Immunol. 26, 388–395.

McCarthy, E.F., 2006. The toxins of William B Coley and the treatment of bone and soft-tissue sarcomas. Iowa Orthop. J. 26, 154–158.

Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., Rot, A., 1997. Transcytosis and surface presentation of IL-8 by venular endothelial cells. Cell 91, 385–395.

Miki, H., Suetsugu, S., Takenawa, T., 1998. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. EMBO J. 17, 6932–6941.

Moynagh, P.N., 2014. The roles of Pellino E3 ubiquitin ligases in immunity. Nat. Rev. Immunol. 14, 122–131.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., Kishimoto, T., 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382, 635–638.

Nauts, H.C., Fowler, G.A., Bogatko, F.H., 1953. A review of the influence of bacterial infection and of bacterial products (Coley's toxins) on malignant tumors in man. Acta Med. Scand. Suppl. 275, 5–103.

Neptune, E.R., Bourne, H.R., 1997. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. Proc. Natl. Acad. Sci. U.S.A. 94, 14489–14494.

Ngadjeua, F., Chiaravalli, J., Traincard, F., Raynal, B., Fontan, E., Agou, F., 2013. Two-sided ubiquitin binding of NF-κB essential modulator (NEMO) zinc finger unveiled by a mutation associated with anhidrotic ectodermal dysplasia with immunodeficiency syndrome. J. Biol. Chem. 288, 33722–33737.

Nourshargh, S., Hordijk, P.L., Sixt, M., 2010. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. Nat. Rev. Mol. Cell Biol. 11, 366–378.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.L., Arenzana, S.F., Schwartz, O., Heard, J.M., Clark-Lewis, I., Legler, D.F., Loetscher, M., Baggiolini, M., Moser, B., 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature 382, 833–835.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi, R.G., Lobb, R., 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59, 1203–1211.

Ostermann, G., Weber, K.S., Zernecke, A., Schroder, A., Weber, C., 2002. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. Nat. Immunol. 3, 151–158.

Park, Y.C., Ye, H., Hsia, C., Segal, D., Rich, R.L., Liou, H.C., Myszka, D.G., Wu, H., 2000. A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. Cell 101, 777–787.

Paterson, Y., Maciag, P.C., 2005. Listeria-based vaccines for cancer treatment. Curr. Opin. Mol. Ther. 7, 454–460.

Pearl, R., 1929. Cancer and tuberculosis. Am. J. Hyg. 9, 97–159.

Pizon, V., Lerosey, I., Chardin, P., Tavitian, A., 1988. Nucleotide sequence of a human cDNA encoding a ras-related protein (rap1B). Nucleic Acids Res. 16, 7719.

Pohlman, T.H., Stanness, K.A., Beatty, P.G., Ochs, H.D., Harlan, J.M., 1986. An endothelial cell surface factor(s) induced in vitro by lipopolysaccharide, interleukin 1, and tumor necrosis factor-α increases neutrophil adherence by a CDw18-dependent mechanism. J. Immunol. 136, 4548–4553.

Pollard, T.D., Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–465.

Proudfoot, A.E., 2006. The biological relevance of chemokine-proteoglycan interactions. Biochem. Soc. Trans. 34, 422–426.

Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M., Suzuki, N., Kato, R., Kensche, T., Uejima, T., Bloor, S., Komander, D., Randow, F., Wakatsuki, S., Dikic, I., 2009. Specific recognition of linear ubiquitin chains by NEMO is important for NF-κB activation. Cell 136, 1098–1109.

Reedquist, K.A., Ross, E., Koop, E.A., Wolthuis, R.M., Zwartkruis, F.J., van Kooyk, Y., Salmon, M., Buckley, C.D., Bos, J.L., 2000. The small GTPase, Rap1, mediates CD31-induced integrin adhesion. J. Cell Biol. 148, 1151–1158.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., Horwitz, A.R., 2003. Cell migration: integrating signals from front to back. Science 302, 1704–1709.

Ruddle, N.H., Waksman, B.H., 1968. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. 3. Analysis of mechanism. J. Exp. Med. 128, 1267–1279.

Santoso, S., Sachs, U.J., Kroll, H., Linder, M., Ruf, A., Preissner, K.T., Chavakis, T., 2002. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. J. Exp. Med. 196, 679–691.

Sen, R., Baltimore, D., 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46, 705–716.

Seymour, R.E., Hasham, M.G., Cox, G.A., Shultz, L.D., Hogenesch, H., Roopenian, D.C., Sundberg, J.P., 2007. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. Genes Immun. 8, 416–421.

Shear, M.J., Andervont, H.B., 1936. Chemical treatment of tumors. III. Separation of hemorrhage-producing fraction of B. coli filtrate. Proc. Soc. Exp. Biol. Med. 34, 323–325.

Shear, M.J., Turner, F.C., 1943. Chemical treatment of tumours. V. isolation of the haemorrhage-producing fraction from *Serratia marcescens* (*Bacillus prodigious*). J. Natl. Cancer Inst. 4, 81–97.

Shimonaka, M., Katagiri, K., Nakayama, T., Fujita, N., Tsuruo, T., Yoshie, O., Kinashi, T., 2003. Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. J. Cell Biol. 161, 417–427.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76, 301–314.

Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M., von Brühl, M.L., Gärtner, F., Khandoga, A.G., Legate, K.R., Pless, R., Hepper, I., Lauber, K., Walzog, B., Massberg, S., 2013. Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. Nat. Immun. 14, 41–51.

Stieglitz, B., Rana, R.R., Koliopoulos, M.G., Morris-Davies, A.C., Schaeffer, V., Christodoulou, E., Howell, S., Brown, N.R., Dikic, I., Rittinger, K., 2013. Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP. Nature 503, 422–426.

Suda, T., Hashimoto, H., Tanaka, M., Ochi, T., Nagata, S., 1997. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. J. Exp. Med. 186, 2045–2050.

Suetsugu, S., Kurisu, S., Oikawa, T., Yamazaki, D., Oda, A., Takenawa, T., 2006. Optimization of WAVE2 complex-induced actin polymerization by membrane-bound IRSp53, PIP(3), and Rac. J. Cell Biol. 173, 571–585.

Takada, H., Chen, N.J., Mirtsos, C., Suzuki, S., Suzuki, N., Wakeham, A., Mak, T.W., Yeh, W.C., 2003. Role of SODD in regulation of tumor necrosis factor responses. Mol. Cell Biol. 23, 4026–4033.

Takahashi, N., Vereecke, L., Bertrand, M.J.M., Durpez, L., Berger, S.B., Divert, T., Goncalves, A., Sze, M., et al., 2014. RIPK1 ensures intestinal homeostasis by protecting the epithelium against apoptosis. Nature 513, 95–99.

Tamatani, M., Che, Y.H., Matsuzaki, H., Ogawa, S., Okado, H., Miyake, S., Mizuno, T., Tohyama, M., 1999. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. J. Biol. Chem. 274, 8531–8538.

Toniolo, C., Bonora, G.M., Showell, H., Freer, R.J., Becker, E.L., 1984. Structural requirements for formyl homooligopeptide chemoattractants. Biochemistry 23, 698–704.

Topolian, S.L., Hodi, S.F., Brahmer, J.R., Gettinger, S.N., Smith, D.C., et al., 2012. Safety, activity and immune correlates of anti-PD-1 antibody in cancer. New Engl. J. Med. 366, 2443–2454.

Van Keymeulen, A., Wong, K., Knight, Z.A., Govaerts, C., Hahn, K.M., Shokat, K.M., Bourne, H.R., 2006. To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front. J. Cell Biol. 174, 437–445.

Vandenabeele, P., Galluzzi, L., van den Berghe, T., Kroemer, G., 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. 11, 700–714.

Verstrepen, L., Bekaert, T., Chau, T.L., Tavernier, J., Chario, A., Beyaert, R., 2008. TLR-4, IL-R1 and TNF-R signaling to NF-kB: variations on a common theme. Cell Mol. Life Sci. 65, 2964–2978.

Wajant, H., Pfizenmaier, K., Scheurich, P., 2003. Tumor necrosis factor signaling. Cell Death Differ. 10, 45–65.

Waller, A., 1846a. Microscopic examination of some of the principal tissues of the animal frame, as observed in the tongue of the living frog, toad etc. Phil. Mag. 29, 271–287.

Waller, A., 1846b. Microscopic observations on the perforation of the capillaries by the corpuscles of the blood, and on the origin of mucus and pus-globules. Phil. Mag. 29, 397–405.

Wright, S.D., Silverstein, S.C., 1982. Tumor-promoting phorbol esters stimulate C3b and C3b′ receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. 156, 1149–1164.

Yang, S., Wang, B., Tang, L.S., Siednienko, J., Callanan, J.J., Moynagh, P.N., 2013. Pellino3 targets RIP1 and regulates the pro-apoptotic effects of TNFα. Nat. Commun. 4, 2583.

Zhang, J., Clark, K., Lawrence, T., Peggie, M.W., Cohen, P., 2014. An unexpected twist to the activation of IKKβ: TAK1 primes IKKβ for activation by autophosphorylation. Biochem. J. 461, 531–537.

Zigmond, S.H., 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J. Cell Biol. 75, 606–616.

Zlotnik, A., Yoshie, O., 2000. Chemokines: a new classification system and their role in immunity. Immunity 12, 121–127.

Zwartkruis, F.J., Bos, J.L., 2000. Ras and Rap1: two highly related small GTPases with distinct function. Exp. Cell Res. 1253, 157–165.

C H A P T E R

15

Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases

THE FAMILY OF NON-RECEPTOR PROTEIN TYROSINE KINASES

There is an important family of receptors that, while possessing no intrinsic catalytic activity, induce responses similar to those of the receptor tyrosine kinases. These operate by recruiting extrinsic protein tyrosine kinases (nrPTKs) that exist either as soluble components in the cytosol or as membrane-associated entities. They can be divided into subgroups based on the sequence of their kinase domains or the presence of additional domains (PH, FCH, etc.) (Figure 15-1) (Robinson et al., 2000). Recruitment of non-receptor PTKs and the tyrosine phosphorylations that ensue are usually the first steps in the assembly of substantial signaling complexes ("signalosomes"), consisting of a dozen or more components that bind and interact with each other.

Examples of the class of receptors that recruit non-receptor PTKs include those that mediate immune responses:

- **The T-lymphocyte receptor (TCR)**. Involved in detection of processed antigens presented in the context of the major histocompatibility complex (MHC). Subsequently, it regulates the clonal expansion of T-cells (Cantrell, 1996).
- **The B-lymphocyte receptor (BCR)**. Involved in the detection of non-processed antigens. Important for the elaboration of antigen-specific immunoglobulins necessary for defense against infection by microorganisms (Burg et al., 1994).
- **The interleukin-2 receptor (IL2RA/B)**. The cytokine IL2, secreted by a subset of T-helper cells, enhances the proliferation of activated T- and B-cells and increases the cytolytic activity of natural killer (NK) cells and the secretion of IgG.

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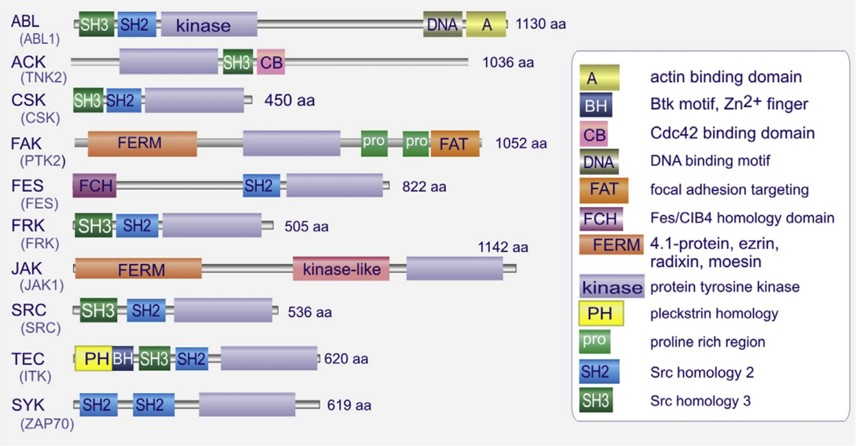


FIGURE 15-1 Non-receptor protein tyrosine kinase members. The non-receptor tyrosine protein (nrRTK) kinases are subdivided into 10 groups, most of which contain SH2 and SH3 domains. Several were originally discovered as transforming genes of viral genomes, hence names such as Src or Abl, derived from Rous sarcoma virus or Abelson murine leukemia virus. *Adapted from Robinson et al. (2000).*

- **The high affinity (epsilon) receptor for IgE (FCER1A)**. Present on mast cells and blood-borne basophils (Metzger, 1992). This plays an important role in hypersensitivity and in the initiation of acute inflammatory responses.
- **Erythropoietin receptor (EPOR)**. The cytokine erythropoietin (EPO) plays an important role in the final stage of maturation of erythroid progenitor cells into mature red blood cells (Syed et al., 1998). For this reason, it has been abused by athletes aiming to boost their performance in endurance sports such as cycling. The receptor is also present on other cell types suggesting that EPO may have effects on other tissues, still to be discerned.
- **Prolactin receptors**. Primarily involved in the regulation of lactation. In addition, prolactin has been implicated in the modulation of immune responses. For instance, it regulates the level of NK-mediated cytotoxicity. It has also gained attention as a potential male contraceptive.
- **Other non-receptor PTKs**. These are discussed in Chapter 11, "Signal Transduction to and from Adhesion Molecules," where they are shown to play pivotal roles in cell survival and proliferation.

Web resources

Information about members of the non-receptor protein kinase can be found in a dedicated Wiki article URL: http://en.wikipedia.org/wiki/Non-receptor_tyrosine_kinase.

continued

Tyrosine protein kinases do not constitute a separate family in the HUGO gene nomenclature database. Depending on the protein domains they carry, they belong to different gene families. Most of the non-receptor tyrosine protein kinase members (21 out of 31) can be found in the "SH2 domain-containing" family of proteins. URL: http://www.genenames.org/cgi-bin/genefamilies/set/741.

T-CELL RECEPTOR SIGNALING

T-lymphocytes are central to cell-mediated immunity. When activated, they proliferate and differentiate, becoming either cytotoxic, helper T-cells, or long-lasting memory cells. Cytotoxic T-cells kill specific targets, most commonly virus-infected cells while helper T-cells assist other cells of the immune system, mainly B-lymphocytes. Effective T-cell activation requires the coordinated attentions of several different ligands with their receptors. This is not the only unusual aspect. The primary ligands that activate T-cells are not classical first messengers (i.e., blood-borne soluble molecules) but proteins presented on the membranes of neighboring cells. For example, the stimulation of T-lymphocytes that are naïve to any previous form of activation involves the T-cell receptor (Figure 15-2(a)). This detects foreign proteins (antigens) in the form of short peptides that are proffered on the surface of the target cells. An infected cell, expressing viral proteins, presents fragments fixed in a groove on the MHCI (major histocompatibility complex type-I). MHCI molecules are expressed on virtually all nucleated cells circulating antigens obtained through phagocytosis (or endocytosis) are processed by specialized cells such as dendritic cells, B-lymphocytes, and macrophages and, which then present protein fragments on their surfaces, again attached to an antigen-presenting molecule, this time MHC Class II (Figure 15-2(b) and (c)). Here, the main function of the T-cell is to help the target cell, the B-lymphocyte, to make antibodies.

More than one lymphocyte receptor must be engaged to ensure activation

The cell–cell interaction necessary for T-cell activation requires intimate contact, calling on specialized adhesion molecules, as well as three quite distinct ligand–receptor interactions (Figure 15-2(a)). It is the combination of antigen and MHC that initiates the T-cell response, but there are two possible outcomes. Only in the presence of a second, "co-stimulatory" signal do the cells become fully activated. The full response comprises transcription of early response genes, followed by the synthesis and release of

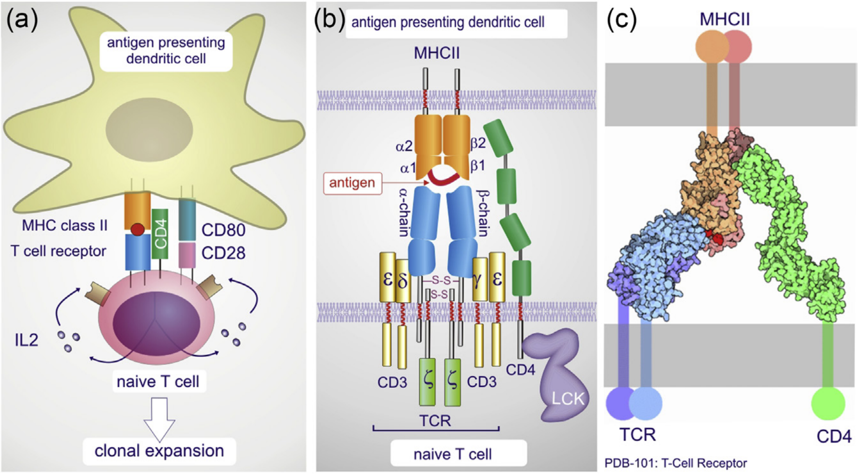


FIGURE 15-2 Clonal expansion of naive T-lymphocytes. (a) Clonal expansion is signaled through binding of an antigen, presented in the groove of the MHC II on an antigen presenting dendritic cell (above), to the receptor (TCR) on the naive T-cell (below). Altogether, the additional interaction of a number of co-stimulatory adhesion molecules, such as CD4, CD80 (formerly B7), and CD28, results in the production of IL2 along with its receptor on the T-cell. This induces a proliferation signal that is responsible for the clonal expansion of those T-lymphocytes that exclusively recognize this particular antigen. (b) The TCR is a disulfide-linked heterodimer of α and β chains. These have hypervariable regions that detect the antigen, presented as a short peptide in the groove of an MHC molecule. This heterodimer forms a complex with six polypeptides, two ζ chains and the δ, ε, and γ chains of the CD3 complex. A CD4 or CD8 molecule is also associated with the TCR in helper or cytotoxic T-cells, respectively. Importantly, attachment of CD4 (or CD8) to the MHC chain brings the non-receptor PTK LCK into the vicinity of the T-cell receptor. (c) Realistic representation of the TCR/MHC interaction. *Image produced by David Goodsell, the Scripps Research Institute, La Jolla. You can explore the "T-cell receptor" through PDB101 (www.rcsb.org).*

the cytokine IL2, entry into the cell cycle, and differentiation into an effector or memory cell. In the absence of a co-stimulatory signal, the T-cell becomes unresponsive or anergic. Anergy may also be induced by signals, such as PD-1, from tissue-resident cells (induction of lymphocyte tolerance) (Saibil et al., 2007).

Initial cell–cell contact involves low-specificity interaction between CD80 (formerly B7) (on the antigen-presenting cell) and CD28 (on the T-cell). The α and β chains of the T-cell receptor (TCR) may then bind to the peptide presented by the antigen-presenting cell. The TCR is associated with CD3 as components of a complex of six polypeptides, all of which span the membrane (Figure 15-2(b)). The TCR/CD3 bound to MHCII assemble as microclusters, comprising 11–150 copies of each that converge to form the so-called "immunological synapse"

(Dustin et al., 2006). By the recruitment of non-receptor PTKs, components of the TCR/CD3 complex are rapidly phosphorylated. In addition, there is activation of PLCγ1 (PLCG1) with the production of IP_3 and diacylglycerol and elevation of cytosol Ca^{2+}. Thus, the consequences of receptor ligation are not dissimilar from those induced by the receptors for EGF or PDGF.

An early candidate explaining the induction of tyrosine kinase activity emerged with the discovery of LCK ($p56^{lck}$). This T-cell-specific kinase of the Src family (Gacon et al., 1984; Marth et al., 1985) (see Figure 15-1 and Table 15-4) is associated with the membrane and with the cytosolic tail of CD4 (in helper T-cells) or CD8 (in cytotoxic T-cells) (Barber et al., 1989) (Figure 15-2(b)). The extracellular domains of CD4 (or CD8) attach to the MHC, strengthening the rather weak interaction established between the TCR and antigen. They also bring CD4 (or CD8) into the vicinity of the TCR complex, leading LCK to its targets on their ζ and other CD3 chains (the CD3-ζ chain is classified in HUGO as CD247) (Palacios and Weiss, 2004).

TCR-receptor signal complex formation

In resting cells, LCK exists in a primed state. This has an open conformation, accessible to substrate but lacking the activating phosphorylation of residue Y394 in its activation segment. The open state of LCK associated with CD4 (or CD8) is maintained by CD45, a transmembrane phosphatase that targets pY505 in the C-terminal tail. As with SRC, CSK is the kinase that drives LCK into its inactive state (Hermiston et al., 2003; Okada, 2012; Okada et al., 1991) (see Figure 15-13).

Activation of primed LCK is by transphosphorylation by another LCK molecule present in the TCR microcluster. It can then phosphorylate the δ, γ, ε, and ζ chains of CD3 (Cantrell, 1996). The target tyrosines are confined to the so-called ITAM motifs. Phosphorylation of ITAMs (immunoreceptor tyrosine activation motifs) provides multiple docking sites for SH2 domain-bearing molecules, among them ZAP70, a tyrosine kinase, binds to the ζ chain of CD3 (Neumeister et al., 1995) (Figure 15-3). ZAP70 is now phosphorylated by LCK on Y315 and Y319 in the interdomain B segment, and on Y492 in the activation segment, and thereby activated. It in turn causes the phosphorylation of at least two substrates LAT and LCP2 (formerly SLP76). The sequence of events then follows a pattern in which phosphotyrosines bind proteins containing SH2 or PTB domains that may themselves be PTKs and that can phosphorylate and recruit yet more proteins in succession. At each stage, there is the opportunity for the pathways to diverge, involving a range of effectors (Palacios and Weiss, 2004).

TCR ACTIVATION AND FYN

The ITAM motifs in the δ, γ, ε, and ζ chains of CD3 are also targets of FYN ($p59^{fyn}$), another member of the Src-kinase family. FYN is also activated by phosphorylation but its mode of recruitment to the TCR microclusters is not yet understood. FYN is required for efficient TCR signaling during antigen presentation, but it plays a more important role in the survival of naive T-cells (Palacios and Weiss, 2004).

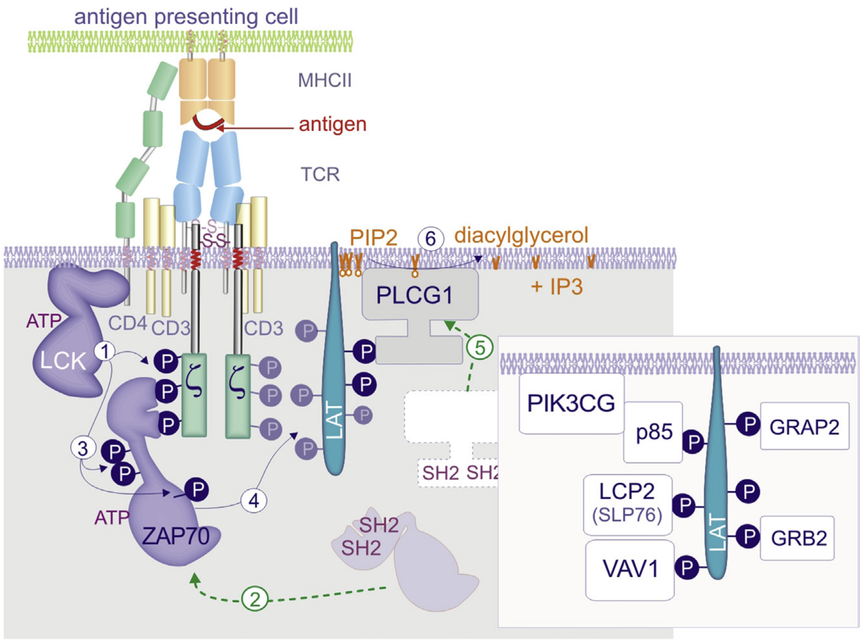


FIGURE 15-3 Signaling complex formation at the TCR complex. The antigen/MHC-bound TCR activates LCK that phosphorylates the ITAM motifs present in the ζ chains of CD3 (1). The resulting phosphotyrosine residues form a docking site for the SH2 domains of ZAP70 (2), another nrPTK. ZAP70 is phosphorylated by LCK in the linker region between the SH2 domains and in the activation segment (3). The activated ZAP70 then phosphorylates several (maximally nine) tyrosine residues on the transmembrane adaptor LAT (4). Various proteins attach. These include PLCG1 (5) which upon attachment becomes phosphorylated and activated by yet another protein kinase ITK (not shown). Others proteins attaching to LAT include the guanine nucleotide exchange factor Vav; the adaptors GRB2, GRAP2, and LCP2 (formerly SLP76); and the p85 regulatory subunit bound to PI3-kinase (PIK3CG). All play important roles in the activation of the IL2 gene. Generation diacylglycerol and IP_3 (6) by PLCG1 is a starting point for the two signaling pathways described in more detail in this section.

One important branch-point is offered by the integral membrane protein LAT that presents no less than nine substrate tyrosine residues (Zhang et al., 1998). When phosphorylated, these recruit a broad range of signaling molecules that include GRAP2, GRB2, LCP2 (formerly SLP76), PLCG1, PI3-kinase-γ (PIK3CG, attached through its p85 regulatory subunit), and the guanine nucleotide exchange factors DBL and VAV1 (Figure 15-3).

The formation of a signaling complex around the TCR and the branching pathways that emanate from it resemble the mechanisms used by the growth factors. However, not all the destinations of these pathways are clear. We outline two mechanisms: one involving calcineurin and the other PKCθ (Figure 15-4). An important starting point is the production of diacylglycerol and IP_3 by PLCγ1.

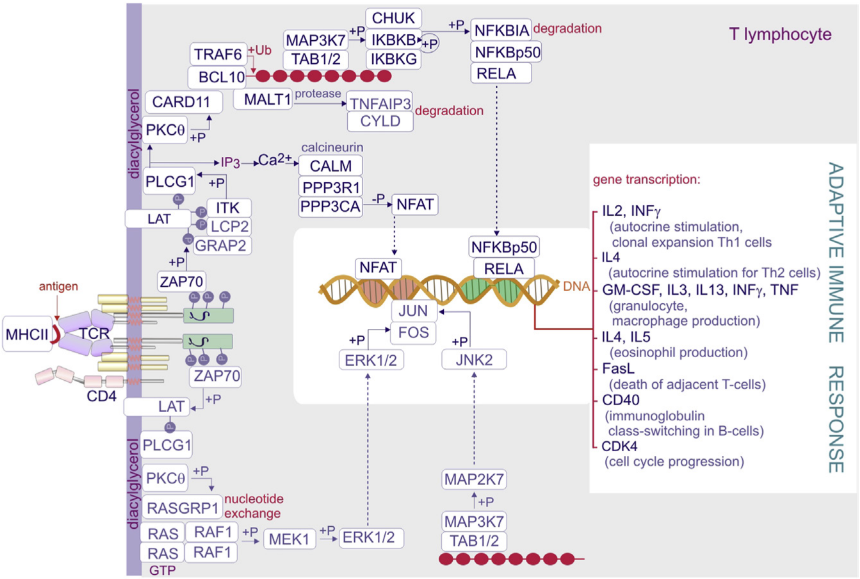


FIGURE 15-4 Overview of signaling pathways downstream of the TCR. The second messengers diacylglycerol and IP_3 activate a number of signaling pathways of which two are elaborated in this chapter. One involves IP_3-released Ca^{2+} results in the activation of the S/T phosphatase calcineurin and leads to activation of NFAT. The other involves the diacylglycerol-mediated activation of PKCθ, which phosphorylates CARD11. The unfolded protein acts as a docking site for the assembly of a huge MALT/TRAF6-ubiquitin ligase complex that causes activation of IKBKB (IKKβ) and then nuclear translocation of NFKB. The genes regulated by NFAT and NFκB are involved in various aspects of the immune response. The production of diacylglycerol also leads to the activation of RASGRP1, a RAS-nucleotide exchange factor, which initiates a MAP-kinase pathway. Finally, TRAF6-mediated ubiquitinylation also recruits TAB1/2 associated with MAP3K7, and this leads to the activation of another MAP-kinase pathway ending with JNK2. Collectively, these pathways induce expression of numerous cytokines and cell surface molecules that play a role in starting the adaptive immune response (clonal expansion of the appropriate T-lymphocytes).

PLCG1 to NFAT

Phosphorylated LAT recruits PLCG1 to the TCR/CD3 complex (Braiman et al., 2006) and then it recruits PI3-kinase-γ (PIK3CG). PI3-kinase plays a role in the activation of phospholipase C because its production of phosphatidyl-3,4,5-triphosphate (PIP3) serves as a first docking site for the protein kinase ITK, interleukin-2-inducible T-cell kinase, a member of the TEC family of protein kinases (return to Figure 15-1). This interaction occurs through the PH domain of the protein kinase. It also carries an SH3 domain with which it interacts with a proline-rich region (PPR) in LCP2, and it carries an SH2 domain that binds to phosphorylated LCP2 (or LAT). Being firmly attached, LCK next phosphorylates ITK in the activation segment (Y512), and this is followed by an autophosphorylation on Y180 (near the SH3 domain) (Andreotti et al., 2010). PLCG1 is next activated through phosphorylation by ITK on Y783. Besides phosphorylation, direct contact between the tyrosine protein kinase domain and the nonclassical SH2 domain (the second, C-terminal, domain, see Figure 15-6) of PLCG1 is also required for activation (Min et al., 2009). Loss of any of these interactions does not stop the TCR response, but they cause a substantially reduction in signal strength. The consequent production of diacylglycerol and IP_3, and the liberation of Ca^{2+} from intracellular stores, leads to the activation of calcineurin (PPP3CA). This serine/threonine phosphatase is associated with two Ca^{2+} sensors, calmodulin (CALM) and calcineurin B (PPP3R1) (Figure 15-5). Calcineurin dephosphorylates numerous phosphoserine residues in the N-terminal segment of the transcription factor NFAT, thereby exposing the nuclear localization signal (Okamura et al., 2000). For a full appreciation of phospholipase C, return to Chapter 3, "Regulation of Muscle Contraction by Adrenoreceptors," Figures 3-15–3-18. The action of Ca^{2+} is discussed in more detail in Chapter 6, "Intracellular Calcium."

Multiple protein kinases and the multiple phosphorylation sites on NFAT. The N-terminal segment of NFAT contains numerous serine phosphorylation sites (about 14), which reside in short-conserved motifs, serine-rich regions (SRR), and serine/proline motifs (SP1, SP2, and SP3). These serine residues surround the NLS. Phosphorylation occurs in the nucleus. DYRKA initially phosphorylates the SP3 site, and this opens the way to further phosphorylations by casein kinase (CK1) and glycogen synthase kinase (GSK3β). Phosphorylation leads to the masking of the NIS and unmasking of the nuclear export signal NES (Gwack et al., 2006) (see Figure 15-6). NFAT1 is actively transported out of the nucleus by exportin-1/RanGTP.

Two calcium waves. An initial wave of Ca^{2+} concentration due to IP_3 mediate release from the endoplasmic reticulum is followed by influx of extracellular calcium ions through plasma membrane channels. This amplification of the Ca^{2+} signal is required for expression of NFAT-controlled genes. Loss of function of the second wave (named CRAC) causes immune deficiency (Feske et al., 2006). Return to Chapter 6, "Intracellular Calcium," Section "Replenishing the intracellular Ca^{2+}-stores."

Nuclear translocation of NFAT is essential for clonal expansion of T-cells because of its pivotal role in the induction of IL2 and IFNγ (for Th1 cells) or IL4 (for Th2 cells). Full transcriptional activity of NFAT only occurs after phosphorylation by ERK1 (or -2) (MAPK3, -1) or p38α (MAPK14) (Avots et al., 1999). In order to drive full expression of IL2, NFAT must associate with the AP1 complex and with NFKB (see later). The domain architecture and interactions of proteins involved in this pathway are illustrated in Figure 15-6.

CALCINEURIN, NFAT, AND ISLET DEVELOPMENT IN THE PANCREAS

Cyclosporin A, a cyclic peptide of 11 residues, of fungal origin, has been applied following transplantation surgery to suppress tissue rejection. However, it was found that it can raise the concentration of blood sugar, effectively causing diabetes mellitus, due to the loss of the insulin-producing β cells in the pancreatic islets of Langerhans (Weir and Fink, 1999). This raised the possibility that calcineurin, being the target of cyclosporin, might play a normal role in adaptive islet responses. Mice deficient in the calcineurin-regulatory subunit (Cnb1/PPP3R1) develop age-related diabetes due to loss of β cells. Conditional expression of active NFAT1 in these β cells rescues the defect and prevents diabetes. NFAT1 regulates transcription of genes critical for β-cell endocrine function. Loss of their expression is linked to type-2 (late-onset) diabetes (Heit et al., 2006).

The PLCG1 to NFKB pathway

In essence, this process involves the assembly of a big MATL1 and an E3-ubiquitin ligase complex, followed by K63-type ubiquitylation of a number of proteins, essentially as described for the toll-like receptors and the TNF receptors (Chapter 13, "Activation of the Innate Immune System" and Chapter 14 "Chemokines and Traffic of White Blood Cells").

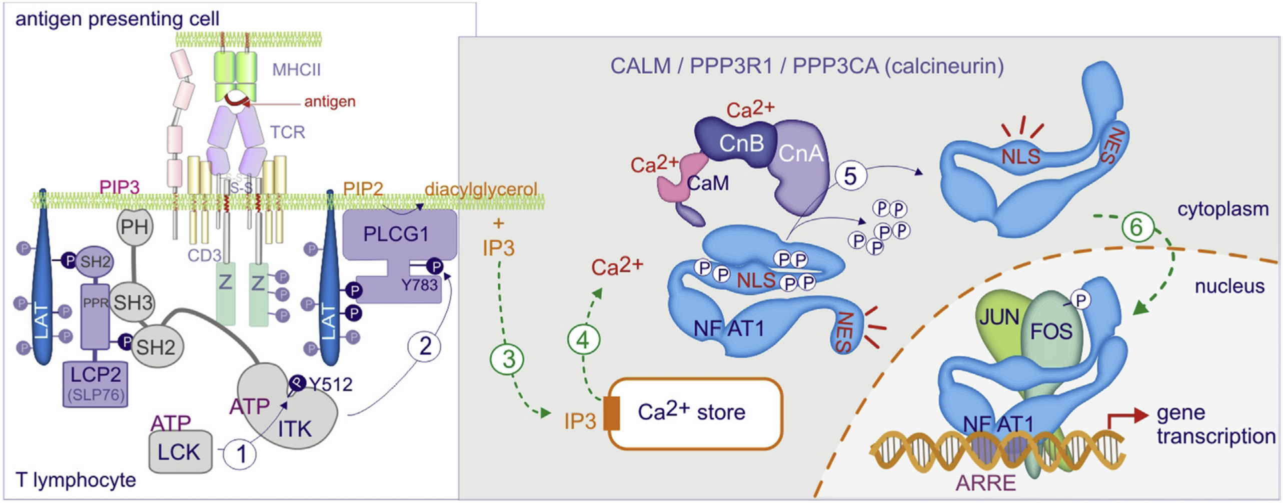


FIGURE 15-5 Calcineurin-mediated activation of NFAT1. Phosphatidyl-3,4,5-trisphosphate (PIP3) brings the tyrosine protein kinase ITK to the membrane through its PH domain. Its SH3 domain interacts with a proline-rich region in LCP2, and its SH2 domain interacts with one of the phosphotyrosine residues. LCK phosphorylates (on Y512) and activates ITK (1), which, in turn, phosphorylates (on Y783) and activates phospholipase Cγ (PLCG1) (2). Hydrolysis of phosphatidyl-4,5-bisphosphate (PIP2) into diacylglycerol and IP_3 (3) leads to IP_3-mediated release of Ca^{2+} from intracellular stores (4). The elevated Ca^{2+} binds to calmodulin (CaM/CALM) and calcineurin B (CnB/PPP3R1), leading to the activation of the catalytic subunit of the serine/threonine phosphatase calcineurin (PPP3CA). Dephosphorylation of numerous phosphoserine residues in the N-terminal segment of NFAT1 ensues, exposing the nuclear localization signal (NLS) and masking of the nuclear export signal (NES). NFAT enters the nucleus and associates with JUN and FOS (AP-1 complex) to drive gene expression.

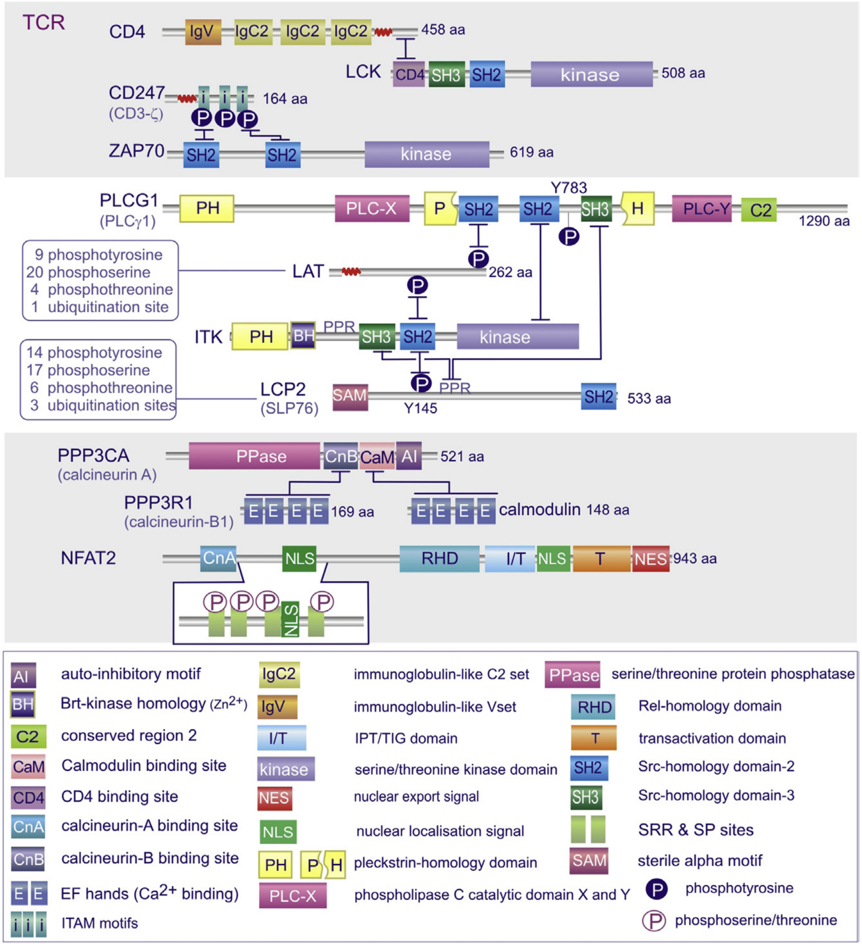


FIGURE 15-6 Domain architecture of proteins involved in TCR-mediated activation of NFAT. A variety of proteins associate with the TCR. They are brought together through a cascade of phosphotyrosine–SH2 interactions. An important signal is transmitted via phospholipase Cγ, which, through activation of calcineurin, results in the nuclear translocation of NFAT2.

These events lead to the destruction of the inhibitor NFKBIA (IκB) and hence lead to the nuclear translocation of the transcription factors NFKBp50/RELA. The assembly process involves the CARD domain, first discovered in the recruitment and activation of caspases (caspase activation recruitment domain). Caspases are proteases involved in programmed cell death, but they also play a role in maturation through the partial proteolysis of cytokines such as pro-IL1B and pro-IL18. All depends on the activation of PKCθ by diacylglycerol in the lipid raft supporting the

TCR–CD3 complex (see Section "The lipid raft hypothesis"). Note that PKCθ, being a member of the novel (or n-) PKC subfamily lacking a functional C2 domain, does not require Ca^{2+} for its activation (return to Chapter 9, "Protein Kinase C in Oncogenic Transformation and Cell Polarity," Figure 9-2). Activated PKCθ phosphorylates the membrane-bound adaptor CARD11 (formerly CARMA1), which causes it to unfold at the interface between two segments: the CARD/CC and MAGUK (the latter of which is composed of PDZ, SH3, and GUK domains). The MAGUK segment attaches to the membrane, whereas the CARD/CC segment recruits the CARD-domain protein BCL10. This leads to filamentous assembly of CARD domains into a macromolecular complex referred to as the CARD11/BCL10/MALT signalosome (Figure 15-7) (Qiao et al., 2013). The CARD assembly process resembles what we have discussed previously for the death-domain (DD) assembly processes (return to Chapter 13, "Activation of the Innate Immune System: the Toll-Like Receptor and Signaling through Ubiquitinylation," Section "Signaling through the TLR4 receptor," Figure 13-5). It is also reminiscent of the nucleation process described for microtubules, where a γ-tubulin ring serves as a scaffold (primer) for the cylindrical assembly of α- and β-tubulin dimers (Kollman et al., 2011). Multiple copies of phosphorylated CARD11 take the role of the primer.

BCL10 is associated with MALT1, a caspase-like protease (a paracaspase) (Uren et al., 2000), and it recruits the TRAF6 E3–ubiquitin ligase complex (comprising TRAF6 and the E2-conjugating proteins UBE2N and UBE2V2) (Lucas et al., 2001). The inclusion of MALT into the complex causes mono-ubiquitinylation and activation, and this is followed by the partial degradation of TNFAIP3 (formerly A20) (Pelzer et al., 2013). This ubiquitin-editing enzyme carries two qualities: it acts as protease, breaking K63-bonds thereby counteracting the activity of TRAF6, and it has E3-ubiquitin ligase activity, causing the formation of K48 ubiquitin chain on E2-conjugating enzymes, leading to their destruction. Another target of MALT1 is CLYD, which is a K63-bond-breaking protease (Rebeaud et al., 2008). Other substrates are BCL10, RELB, and NIK. The recruitment of TRAF6 does the inverse, it brings about K63-type ubiquitylation of TRAF6 itself (on K124, K331) and of BCL10 (on K31, K63) and perhaps other proteins such as CARD11 (on K696, K703), or MALT1 (on K644, K648, K665, etc.), the latter which needs to be mono-ubiquitinylated (on K644) in order to express its protease activity. The multiple K63 ubiquitin chains next invite IKBKG/CHUK/IKBKB, as well as TAB1/TAB2/MAP3K7 complexes into the signalosome (Sun et al., 2004). The recruitment of MAP3K7 allows phosphorylation and activation of IKBKB and this, in turn, phosphorylates NFKBIA (formerly IκB) rendering the inhibitor susceptible to ubiquitylation (K48 type) by the SCF^{BTRC} E3-ubiquitin ligase complex. Ubiquitylated NFKBIA is destroyed by the proteasome.

If the size of the assembled signalosome is not enough, IKBKG (formerly NEMO) is also ubiquitinylated either in a K63- or in a M1-linear manner, and this allows yet further recruitment of the aforementioned complexes thus enhancing the signal (Zhou et al., 2004; Liu et al., 2005).

The liberated NFKB now exposes its nuclear localization signal and translocates to the nucleus where it participates in the induction interleukins together with NFAT and JUN/FOS (AP1 complex). For more detail about these events, see Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation," Section "From TRAF6 to activation of NFKB." For the role of multiple E3-ubiquitin ligases, return to Chapter 14, "Chemokines and Traffic of White Blood Cells," Section "Linear ubiquitinylation is required to stabilize complex I," Figure 14-7.

BCL10, CARD11, and MALT1 in lymphomas. B-cell lymphomas are sometimes due to a lack of apoptosis. The BCL2 gene, an essential component in the activation of pro-caspase-9, was thus discovered (Hockenbery et al., 1991). BCL10 is yet another member of this family (Willis et al., 1999). It promotes apoptosis but also plays an essential role in T-cell activation as discussed here. Loss-of-function mutations are involved in various types of cancers such as malignant mesothelioma and lymphomas. In certain lymphomas, such as diffuse large B-cell lymphoma, missense mutations in the coiled-coil (CC) domain of CARD11 overcome the auto-inhibition and the protein nucleates BCL10 filaments without the requirement of phosphorylation. The NFKB pathway runs at full throttle as a consequence (Lenz et al., 2008). MALT1 came into light as a component of a fusion product caused by chromosomal translocation associated with lymphomas observed in mucosal tissues, hence its name "mucosa-associated lymphoid tissue" (MALT) (Dierlamm et al., 1999). The lymphoma is frequently observed in the stomach (gastric MALT lymphoma) and may arise as a consequence of chronic inflammation caused by *Heliobactor pylori* (the bacterium responsible for stomach ulcers). In these tumors, MATL1 is part of a fusion product with BIRC3 (formerly C-IAP2), an E3-ubiquitin ligase with similar functions as BIRC2 (discussed in Chapter 14, "Chemokines and Traffic of White Blood Cells," Figures 14-4 and 14-7).

As already mentioned, full T-cell activation requires a second, co-stimulatory signal, derived through the engagement of a separate receptor. For some helper T-cells, this is provided by CD28, which binds to the CD80 molecule on antigen-presenting cells. The signaling pathway

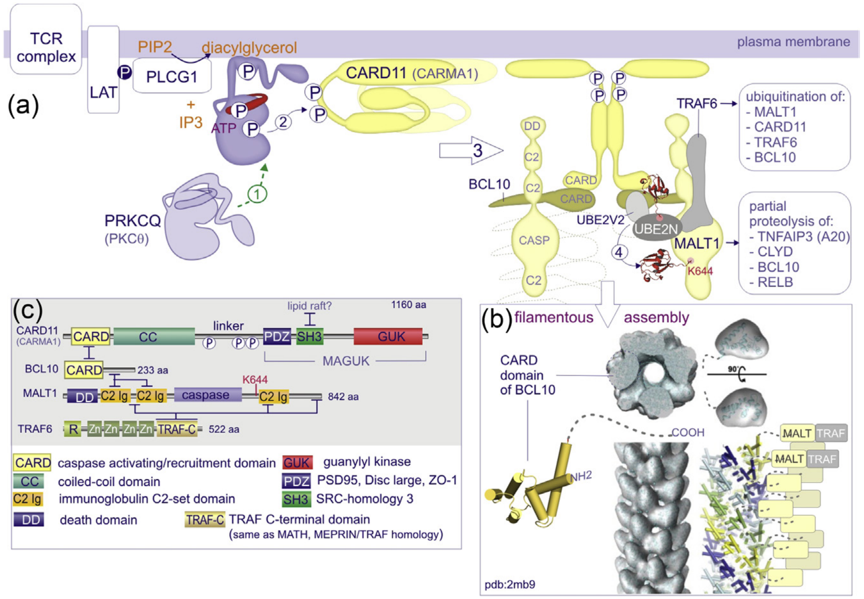


FIGURE 15-7 PLCG1-mediated activation of PKCθ causes activation of the MALT1/BCL10/TRAF6 E3–ubiquitin ligase complex. (a) Diacylglycerol causes membrane attachment and activation of PKCθ (1). This phosphorylates and unfolds the adaptor CARD11 (2). the unfolded CARD11 next binds BCL10 through a CARD-CARD domain interaction. BCL10 in turn is attached to MALT1, a caspase-like protease that cleaves a number of proteins. Two enzymes, TNFAIP3 and CLYD, are ubiquitin-editing enzymes that normally silence ubiquitin signaling by breaking K63-bonds or causing destruction of E2–ubiquitin-conjugating enzymes. MALT1 introduces TRAF6 into the complex, and this together with UBE2V2 and UBE2N causes ubiquitinylation, in a K63- or M1-linear mode, of MATL1 (to activate the protease), CARD11, BCL10, and TRAF6 itself. The ubiquitin chains acts as a docking site for NEMO/IKKα/IKKβ and also the TAB1, -2/MAPK3K7 protein kinase complexes (not shown). (b) The CARD domain of BCL10 is able to assemble into a filamentous structure. Numerous copies of unfolded CARD11, hold together through their coiled-coil region, serve as the nucleation site, in a manner similar to the trimerized TNF receptors that serve as a primer for the assembly of death domains (return to Figure 13-06). *Image adapted from* Qiao et al. (2013). (c) Domain architecture and interactions of CARD11, BCL10, MALT1, and TRAF6. The domain architecture of PKCθ is illustrated in Chapter 9, "Protein Kinase C in Cell Transformation and Cell Polarity," Figure 9-2).

activated by CD28 involves PI3-kinase and mTOR, and it acts to regulate the expression of cyclin E necessary for T-cell proliferation. In fact, prolonged occupation of both TCR and CD28 reduces the requirement of the autocrine IL2 signal (Colombetti et al., 2006). The involvement of mTOR in the co-stimulatory signal explains why rapamycin, an inhibitor of mTOR, acts as a potent inhibitor of T-cell function (immunosuppressant) (Morris, 1991) (subject further discussed in Chapter 16, "Signaling through the Insulin Receptor").

The Ras–MAPK pathway

The production of diacylglycerol and its subsequent activation of PKCθ also result in the phosphorylation and activation of RASGRP1 (Ras guanine release protein), a nucleotide exchange factor that charges RAS with GTP. Two GTP-loaded RAS proteins bind and activate an RAF dimer, a serine/threonine protein kinase, which causes the activation of the MAP-kinase pathway leading to the activation of ERK1/2 (MAPK3 and -1, respectively) (lower section of Figure 15-4). An alternative route occurs through phospho-LAT, which binds the SH2/SH3 adaptor protein GRB2, itself attached to the RAS nucleotide exchange factor SOS1. For detail and explanation, return to Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases,” Figures 10-16 and 10-27.

Finally, the ubiquitin chains brought about by TRAF6 recruit TAB1/2 bound to MAP3K. Besides activating IKBKB, it also phosphorylates and activates MAP2K7, which then phosphorylates and activates JNK2 (MAPK14). The ERK and JNK pathway causes phosphorylation of the transcription factors FOS and JUN, respectively, both of which contribute to gene transcription of cytokines (Figure 15-4, lower section).

For a detailed review about RAS and T-cell activation/thymocyte development, we suggest Kortum et al. (2013).

DOWN-REGULATION OF THE TCR RESPONSE

The strength of the TCR signal depends on the strength of the interaction between receptors and the antigen in the context of the MHCII. It also depends on the number of TCRs that contribute to the formation of microclusters. This requires efficient recycling because as much as 1% of cell surface TCR–CD3 complexes are internalized every minute. Binding of the adapter protein SLA (formerly SLAP1) prevents recycling and dampens the immune response. It binds to the ζ chain of the TCR and recruits CBL (an E3–ubiquitin ligase) into the complex. Subsequent poly-ubiquitylation (K48 type) retains the TCR/CD3 in the endosomal compartment, promoting its degradation in the lysosomes (Myers et al., 2005). A set of reactions regulating the recycling of the EFGR, comparable to those that occur for the TCR ζ chain, is illustrated in Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases,” Figure 10-26.

THE LIPID RAFT HYPOTHESIS

A feature of those cells that are stimulated through immune recognition receptors is the involvement of heterogeneous microdomains, or lipid rafts, present in the plasma membrane. The restriction of certain membrane-associated or transmembrane signaling proteins to particular lipid domains is especially apparent in T- and B-lymphocytes and in mast cells. The existence of such lipid domains appears to be widespread, but knowledge of their wider involvement in signaling mechanisms is lacking and their study has not been without disagreement.

Initially, the discovery of detergent-resistant fractions in membranes obtained from epithelial cells (Simons and Ikonen, 1997) led to the postulation of membrane regions enriched in glycosphingolipids and cholesterol. These have also been referred to as detergent-resistant (or detergent-insoluble) membrane domains or more simply as lipid rafts. They are thought to be phase-separated regions, which, because of the saturated nature of the acyl chains of the component sphingolipids and the presence of cholesterol, possess a higher order of rigidity than the remainder of the membrane. Direct visualization of lipid microdomains in a smooth muscle cell by "single molecule" fluorescence microscopy indicates dimensions ranging between 0.2 and 2 μm at ambient temperatures, but at 37 °C they may be smaller (Schutz et al., 2000). Both their size and density are likely to vary with cell type.

Signaling molecules that appear to be confined to lipid rafts include CD4/CD8 in T-cells and the dual-acylated (N-myristoylated and palmitoylated) non-receptor tyrosine kinases of the Src family. The non-acylated kinase LCK is also raft associated, presumably through the association with CD4/CD8. Other important signaling components located in rafts are LAT and CARD11. Membrane proteins such as the transferrin receptor and the tyrosine phosphatase CD45 are generally absent. The primary receptors, the TCR, the BCR, and the IgE-R (FCER1A), were thought to exist outside rafts until cross-linked during activation, but it now appears that a proportion of these receptors is associated with rafts under resting conditions and that this increases following stimulation. Exactly how lipid rafts function as platforms for the assembly of signaling complexes remains unclear.

SIGNALING THROUGH THE INTERFERON RECEPTORS

Hours to days before the adaptive immune response cuts in to protect against viral infection, α, β, and γ interferons are already at work (Stark et al., 1998). Mice lacking the receptors for these interferons exhibit increased susceptibility to viral infection (van den Broek et al., 1995). The interferons induce the expression of protein kinase-R (dsRNA-dependent protein kinase), 2′-5′ oligoadenylate synthetases (OAS), and methyl transferases. These (respectively) bring about (either directly or indirectly) arrest of protein synthesis, degradation of single-stranded RNA, and inhibition of transcription, thus hindering the propagation of the virus.

In some cases, interferons cause cell death through apoptosis (Stark et al., 1998). IFNα, -β, and -γ also play essential roles in immune modulation. They promote maturation of dendritic cells by increasing surface expression of co-stimulatory molecules and the antigen-presenting complexes (MHCI or MHCII). As a result, the ability of dendritic cells to stimulate the clonal expansion of effector T-cells (such as the Th1-cells, necessary to mount an adaptive immune response) is enhanced. In certain cases, they may induce tolerance (Reis e Sousa, 2006).

Besides their roles in viral defense, IFNα and IFNβ are important in the control of differentiation and growth. Generated in the bone marrow, they affect the differentiation of hematopoietic B-cells, T-cells, osteoclasts, and myeloid dendritic cells (Honda et al., 2005). IFNα2 is used in adjuvant therapy as an anticancer drug. In particular, it has been applied in the treatment of "hairy cell leukemia" (a chronic B-cell leukemia) (Genot, 1994), chronic myelogenous leukemia (CML) (Kantarjian et al., 2006), and to treat some metastasising cancers, such as renal carcinoma and Kaposi's sarcoma, which affects a quarter of AIDS sufferers.

Classification of interferons

Classification of the interferons (IFN), cytokines originally recognized for their antiviral properties (Isaacs and Lindemann, 1957), has been difficult. The interferons are subdivided into interferon-α (IFNA), -β (IFNB1), -γ (IFNG), -κ (IFNK), -λ (IFNL), -ω (IFNW1), and IL6 (formerly interferon-β2). The INFA, -B, -K, and -W (type I) recognize IFNAR1 and IFNAR2. IFNG (type II) binds to IFNGR1 and IFNGR2, whereas IFNL (type III) binds to the IFNLR1/IL10RB receptor dimer. Finally, IL6 binds to IL6R and IL6ST. The receptor for IFNW1 remains enigmatic (for us) (information from HUGO gene nomenclature database and reference (Takaoka and Yanai, 2006)). The structure of IFNα (IFNA) and one of its receptors (IFNAR2) is shown in Figure 15-8.

Web resources

Explore human interferons at the HUGO gene nomenclature database. URLs: for receptors: http://www.genenames.org/cgi-bin/genefamilies/set/599. For the ligands: http://www.genenames.org/cgi-bin/genefamilies/set/598.

Interferon-α receptor and STAT proteins

The interferon-α receptor comprises two subunits, IFNAR1 and IFNAR2, coupled to the FERM domain of the tyrosine protein kinase

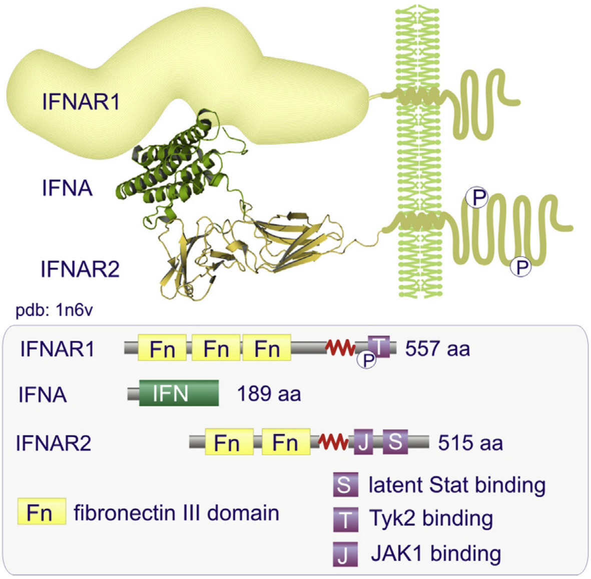


FIGURE 15-8 Interferon-alpha and its receptors. The figure illustrates the organization of IFNAR1 and IFNAR2 bound to IFNα (IFNA). *The shape of IFNAR1 is adapted from the structure presented in Chill et al. (2003).*

TYK2 and JAK1, respectively (van Boxel-Dezaire et al., 2006). Both are members of the Janus kinase family (JAK) of which the domain architecture is shown in Figure 15-1. They are characterized by the possession of a pseudokinase domain, which regulates activity of the second, C-terminal, functional kinase domain. The structure of TYK2 revealed that the two kinase domains interact with their N-terminal lobe and thus may interfere with substrate binding. This explains why they are normally inactive. Most of the reported mutations in cancer-associated alleles cluster in or near this interface and suggest that the oncogenic variant no longer exists in an auto-inhibited state (Lupardus et al., 2014). INFAR2 also binds the latent form of the transcription factors STAT1 or STAT2. To date, seven members of the mammalian family of STATs, all substrates of the JAKs, have been characterized.

Addition of interferon-α2 causes receptor subunit dimerization followed by mutual activation of their associated nrPTKs (Figure 15-9), which then phosphorylate the IFNAR1 receptor subunit. Binding of STAT2 brings the transcription factor into the vicinity of the activated kinases. Phosphorylated STAT2 promotes phosphorylation of STAT1 and, these attached through their SH2 domains to the opposing phosphotyrosine residues,

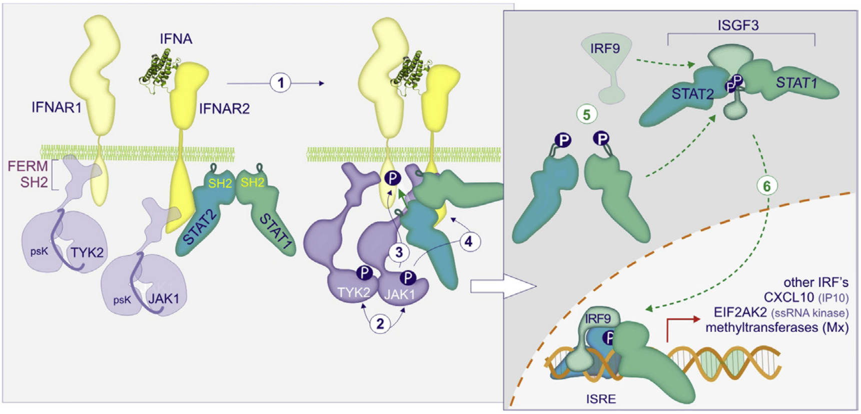
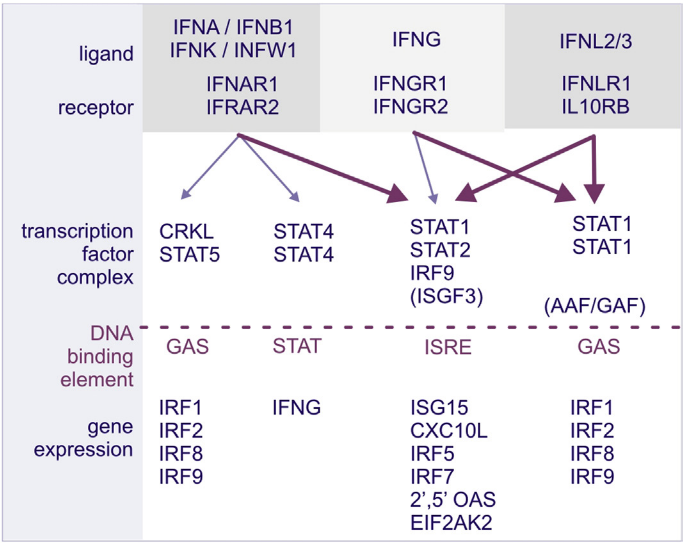


FIGURE 15-9 Activation of STAT transcription factors by the interferon receptor. Binding of interferon-α causes dimerization of the interferon-α receptor (IFNAR1 and -2) (1). This brings together the associated non-receptor tyrosine kinases TYK2 and JAK1, which phosphorylate each other (2) and then IFNAR1 (3). Note that the pseudokinase domain (psK) folds back onto the functional kinase and hinders access of substrate. STAT2, which in its latent form is bound to IFNAR2, associates with the tyrosine-phosphorylated IFNAR1 through its SH2 domain and in doing so, it too becomes phosphorylated (4). The phosphorylated STAT2 catalyses the phosphorylation of STAT1 and these detach from the receptor complex. The dimer is accompanied (5) by an interferon regulatory factor IRF9. The ternary complex, ISGF3, enters the nucleus (6) and binds to DNA at the ISRE. The complex induces expression of genes such as interferon regulatory factors (IRF), ssRNA kinase (EIF21K2), methyltransferases (Mx), and the chemokine CXCL10.

then dissociate from the receptor as a heterodimer. In the cytoplasm, they combine with IRF9. This trimeric complex, named ISGF3, translocates to the nucleus and binds to the DNA at the interferon-stimulated response element (ISRE) (Shual et al., 1993). Among the genes that contain the ISRE are interferon-regulated factors, which amplify the production of type-I interferons, the chemokine CXCL10 (formerly IP10), and also genes coding for the ssRNA-dependent protein kinase EIF2AK2 (formerly PKR), the 2′-5′ oligoadenylate synthetases (OAS), and methyltransferases (Mx), which act collectively to keep the viral infection in check.

An alternative complex, less abundant, and made up of STAT1 dimers can also result from IFNAR1/IFNAR2 signaling. This complex binds to the GAS element and induces the expression of IRFs. For the IFNγ receptors, STAT1 homodimers predominate. The STATs also convey signals issuing from other interferon receptors. The specificity of the intracellular signal is determined by the particular combinations of STAT that are phosphorylated and activated (see Table 15-1).

TABLE 15-1 Diversity in cytokine-induced signaling. The spectrum of downstream STATs and STAT-associated proteins determines the outcome of the cellular response to a particular member of the interferon family. A thick red arrow represents the predominant pathway, and thin blue arrows signify alternative pathways



ligand	IFNA / IFNB1 IFNK / INFW1	IFNG	IFNL2/3
receptor	IFNAR1 IFRAR2	IFNGR1 IFNGR2	IFNLR1 IL10RB

transcription factor complex	CRKL STAT5	STAT4 STAT4	STAT1 STAT2 IRF9 (ISGF3)	STAT1 STAT1 (AAF/GAF)
DNA binding element	GAS	STAT	ISRE	GAS
gene expression	IRF1 IRF2 IRF8 IRF9	IFNG	ISG15 CXC10L IRF5 IRF7 2',5' OAS EIF2AK2	IRF1 IRF2 IRF8 IRF9

Information from Takaoka and Yanai (2006) and Ihle, 1995).

Alternative signaling pathways

The pathway described earlier does not account for all the biological effects of type-I interferons (α, β, κ, ω). Activation of IFNAR1 and -2 also causes phosphorylation of the insulin receptor substrates IRS1 and -2. These then act as docking proteins for SH2-domain-bearing proteins such as the p85/p110 PI3-kinase complex (PIK3R1/PIK3CG). Membrane localization of this complex results in the production of PI-3,4,5-trisphosphate with subsequent activation of the AKT signal pathway (see Chapter 16, "Signaling through the Insulin Receptor," Section "AKT and activation through PI-3,4,5-P3"). This has been described as being both dependent and independent of JAK1/TYK2 (van Boxel-Dezaire et al., 2006), depending on the particular cell type. STAT1 can also be phosphorylated by the serine/threonine protein kinases MEK1 (MAP2K1), ERK2 (MAPK1), p38a (MAPK14), casein kinase 2 (CSNK2A3), or protein kinase C-δ (PRKCD). Inappropriate serine phosphorylation of STAT1 plays a role in the survival of transformed stem cells in Wilms' (kidney) tumors (Timofeeva et al., 2006).

Down-regulation of the JAK–STAT pathway

Nuclear dephosphorylation and recycling of STATs

Dimerized and phosphorylated STATs are rapidly dephosphorylated in the nucleus (Haspel and Darnell, 1999). This is thought to be caused by non-SH2–tyrosine phosphate interactions that force a rearrangement of the dimer partners, so breaking the SH2–tyrosine phosphate bonds and exposing them to nuclear phosphatases (Figure 15-10) (Zhong et al., 2005). The enzymes involved are not clearly established. In order to maintain the system of signaling, the dephosphorylated STATs exit the nucleus to be rephosphorylated at the membrane and return as active dimers. When a large number of cytokine receptors are active, the STAT proteins recirculate rapidly and in consequence, a large proportion is retained as active dimers in the nucleus, stimulating gene transcription. When only few receptors are active, the majority accumulate in the cytoplasm in their inactive state. STAT proteins appear to act as a remote sensor, communicating between the cell surface and the nucleus, though, as described below, the situation is actually more complex (Swameye et al., 2003).

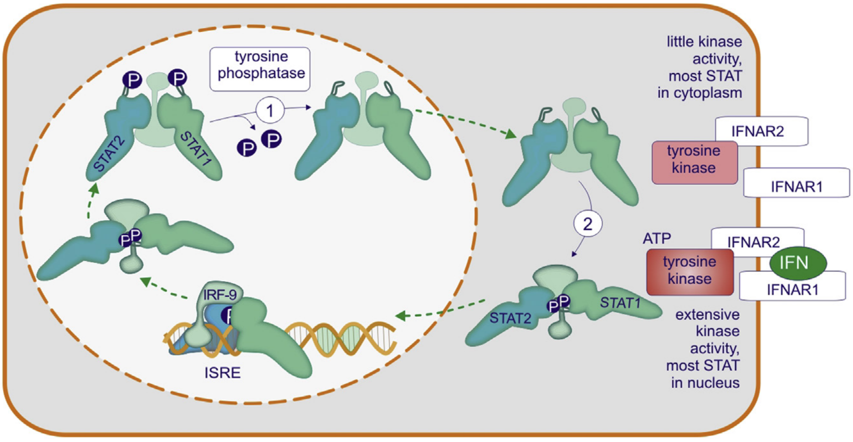


FIGURE 15-10 STAT proteins act as remote sensors. STAT proteins that dissociate from the DNA are dephosphorylated by tyrosine phosphatases (1). They return to the cytoplasm and only reenter the nucleus if they are rephosphorylated by (active) IFN receptors (2). With a large proportion of receptors occupied, the cycle is rapid and STAT accumulates in the nucleus. With few receptors occupied, the cycle is slow, resulting in an accumulation of STAT in the cytoplasm. Effectively, STAT acts as a remote sensor of receptor occupation.

*Dephosphorylation of the IFN*A *receptor-1*

The dual-specificity phosphatases PTPN6 (formerly SHP-1) and PTPN11 (formerly SHP-2) bind the phosphorylated IFNAR1, thereby competing with and dephosphorylating STAT and the tyrosine kinases. The pathway is effectively silenced (Figure 15-11(a)). PTPN6 is mainly expressed in hematopoietic cells, PTPN11 ubiquitously. PTPN11 may also act in the nucleus and participate in the dephosphorylation of nuclear STAT proteins (Wu et al., 2002). Loss of PTPN6 results in hyperphosphorylation of JAK1 and is the cause of immunological and hematopoietic dysfunctions (mice exhibit the "moth eaten" phenotype) (De Luca et al., 1997). Lack of PTPN11 is embryonic lethal. In addition, PTPRC (CD45) is involved as its absence also causes hyperactivation of JAK1. Another cytosolic phosphatase, PTPN1 (formerly PTP1B), has the capacity to dephosphorylate both JAK2 and TYK2. This results in the reduced phosphorylation of IFNAR1 and thus an arrest of STAT signaling (Figure 15-11(b)). Dual specificity and tyrosine phosphatases are further dealt with in Chapter 18, "Protein Phosphatases."

Cytokines, including IL2, IL3, and EPO, induce the "suppressors of cytokine signaling" (SOCS1, -2, and -3). These all carry an SH2 domain with which they bind to the tyrosine-phosphorylated IFNAR1 and, in the case of SOCS1, to tyrosine-phosphorylated JAK1 (Figure 15-11(c)). The prevailing hypothesis for their function is that they inhibit both by competing with STAT binding and by linking the components of the receptor JAK–STAT pathway to an E3–ubiquitin ligase complex. This then leads to ubiquitylation (K48 type) and destruction of JAK1, STAT, or the INFAR1 receptor. Loss or mutation of SOCS is associated with primary mediastinal B-cell lymphoma (Valentino and Pierre, 2006).

Mediastinal large B-cell lymphoma accounts for about 1 in 50 of all cases of non-Hodgkin-type lymphoma. It is a diffuse large B-cell lymphoma deriving from a rare type of B-cell present in the thymus. It occurs most commonly in people between 25 and 40 and is twice as common in women as in men.

STAT signaling without phosphorylation

STAT signaling is also regulated independently of protein phosphorylation. The family of protein inhibitors of activated STATs (PIAS) form complexes with selected STATs and prevent their stimulation of transcription (Figure 15-11(d)) (Liu et al., 1998; Shuai and Liu, 2005).

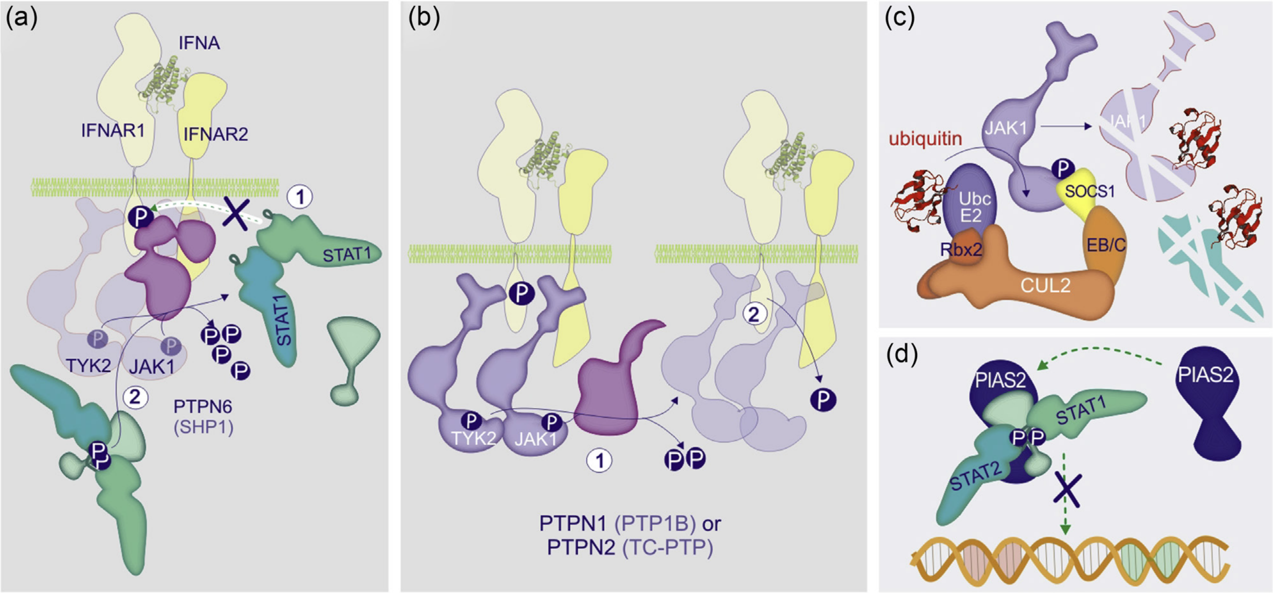


FIGURE 15-11 Inactivation of the IFN pathway. Downregulation of the IFN pathway occurs at different levels. (a) Tyrosine phosphatase PTPN6 binds the phosphorylated IFNAR1 receptor, so preventing association of STAT-2. It dephosphorylates and deactivates STAT-1/2 complexes and also the tyrosine protein kinases associated with the IFN receptors. (b) PTPN1 deactivates TYK2 and JAK1, so preventing further activation of STATs-1 and -2. (c) The SH2 domain of SOCS recognizes tyrosine-phosphorylated IFNAR1 and JAK1. Binding prevents further access of STAT-2 to the receptor and inhibits JAK1 activity. SOCS proteins associate with components of a big E3–ligase complex causing polyubiquitylation (K48) of IFNAR1, JAK1, and STAT proteins. Polyubiquitylated proteins are destroyed by the proteasome. (d) PIAS proteins (example PIAS2) associate with STAT-1/2 and IRF-9 and prevent their association with DNA.

TABLE 15-2 Protein inhibitors of activated STATs

Inhibitor of activated STAT	STAT isotype
PIAS1	STAT1
PIAS3	STAT3
PIAS2 recruits HDAC	STAT4
PIAS4 recruits HDAC	STAT1

PIAS2 and PIAS4 also bind to histone deacetylases and contribute to the reduction of gene transcription by rendering the DNA less accessible to RNA. Loss of PIAS3 and PIAS4 is associated with anaplastic T-cell lymphoma that is characterized by high levels of nuclear STAT3 (Table 15-2).

ONCOGENES, MALIGNANCY, AND SIGNAL TRANSDUCTION

Viral oncogenes

Infection by viruses carrying oncogenes can cause malignant cell growth. Although first recognized as causative agents in avian cancers just over 100 years ago (return to Chapter 1, "Prologue: Signal Transduction from a Historical Perspective," Section "Viruses and tumors"), for much of the twentieth century there was doubt that any human cancers were initiated in this way. Even now, almost all the information in this area refers to nonhuman animals. There are a number of problems here. First of all, as was already apparent in the first decade of the century (Warthin, 1907, 1904; Ellermann and Bang, 1908), demonstration of a viral mode of transmission depends on the induction of disease by transfer of tissue filtrates from animal to animal. Some viruses only become oncogenic as a consequence of multiple passages and through different animal species. Secondly, while there are many human cancers that are certainly associated with viral infection, in most cases it is far from certain whether it is the virus that initiates the condition or whether it is merely permissive of induction by another agent, such as a chemical carcinogen. An exception is the human papilloma virus (HPV), the causative agent of skin warts (Kidd et al., 1936), and epithelial (cervical) cancer. In general, the transforming products of the viral oncogenes behave as persistently activated mutants of (hijacked) endogenous cellular proteins having key regulatory roles in mitogenesis.

HPV: More than 100 different types of HPV exist, most of which are benign. About 30 types are spread by sexual contact and are classified as either low or high risk. Some types of HPV cause genital warts—single or multiple bumps that appear in the genital areas of men and women including the vagina, cervix, vulva (area outside of the vagina), penis, and rectum. Many people infected with HPV have no symptoms. Other types cause common skin warts, such as those found on the hands and soles of the feet. Source: NAID/NIH, USA.

Perhaps the most famous wart in all history sat on the chin of Oliver Cromwell, the Lord Protector of England during the period of the Commonwealth (1653–1658). When commissioning a portrait of himself, he told the painter, Sir Peter Lely: *I desire you would use all your skill to paint my picture truly like me, and not flatter me at all; but remark all the roughness, pimples, warts and everything, otherwise I will never pay a farthing for it.*

The illustration in Figure 15-12 shows an example of how the avian Rous sarcoma oncogene v-Src product (RSVH1) has arisen from a change of the amino acid composition of the C-terminus of SRC. Most significantly, this involves the loss of the most carboxy-terminal tyrosine residue with the result that the mutated protein, v-Src, is unable to fold into its inactive state (compare nucleotide sequence in red). Alternatively viral oncogene products are authentic viral proteins that interfere with the functioning of nuclear proteins such as the tumor suppressors TP53 and RB1. Examples are the transforming antigens (T-antigens) of DNA papovaviruses such as Simian vacuolating virus SV-40 (primate), BK (human), or JC (human) (De Luca et al., 1997; Doherty and Freund, 1997).

Soon after its discovery in 1960, Simian vacuolating virus-40 (SV40) was found as a contaminant in poliovirus vaccine. Clearly, this was a great big headache. Already, more that 98 million Americans (and countless more throughout the world) had received one or more doses of the vaccine during the period (1955–1963). Could immunization cause cancer? As luck turned out, nothing came of it. No widespread incidence of SV40 infection in the population, no increase in tumors nor any direct role for SV40 in human cancer (Poulin and DeCaprio, 2006).

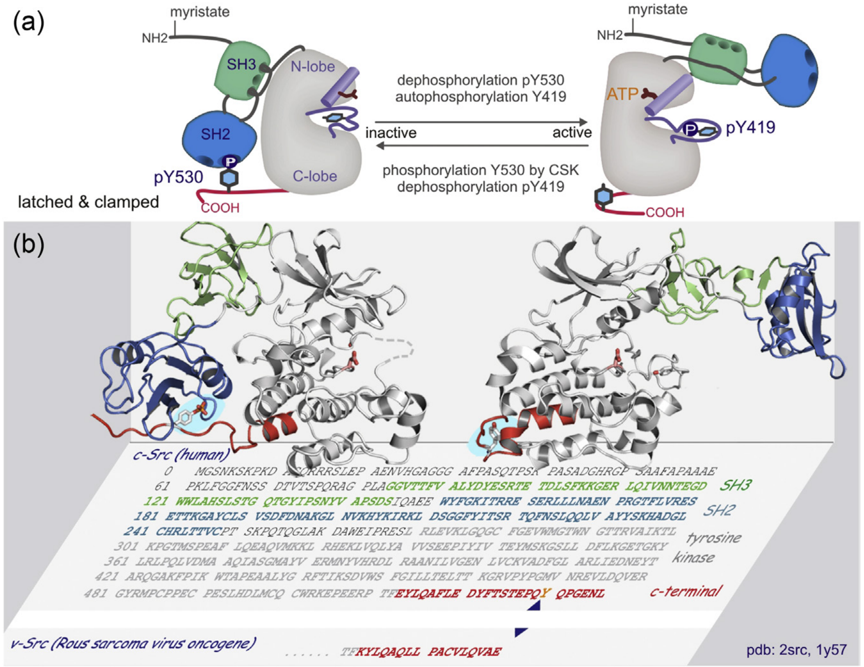

(a)
myristate
NH2
SH3
N-lobe
SH2
P
pY530
C-lobe
latched & clamped
COOH
dephosphorylation pY530
autophosphorylation Y419
inactive
active
phosphorylation Y530 by CSK
dephosphorylation pY419
myristate
NH2
ATP
P
pY419
COOH
(b)
c-Src (human)
0 MGSNKSKPKD ASQRRRSLEP AENVHGAGGG AFPASQTPSK PASADGHRGP SAAFAPAAAE
61 PKLFGGFNSS DTVTSPQRAG PLAGGVTTFV ALYDYESRTE TDLSFKKGER LQIVNNTEGD
SH3
121 WWLAHSLSTG QTGYIPSNYV APSDSIQAEE WYFGKITRRE SERLLLNAEN PRGTFLVRES
SH2
181 ETTKGAYCLS VSDFDNAKGL NVKHYKIRKL DSGGFYITSR TQFNSLQQLV AYYSKHADGL
241 CHRLTTVCPT SKPQTQGLAK DAWEIPRESL RLEVKLGQGC FGEVWMGTWN GTTRVAIKTL
tyrosine
301 KPGTMSPEAF LQEAQVMKKL RHEKLVQLYA VVSEEPIYIV TEYMSKGSLL DFLKGETGKY
kinase
361 LRLPQLVDMA AQIASGMAYV ERMNYVHRDL RAANILVGEN LVCKVADFGL ARLIEDNEYT
421 ARQGAKFPIK WTAPEAALYG RFTIKSDVWS FGILLTELTT KGRVPYPGMV NREVLDQVER
481 GYRMPCPPEC PESLHDLMCQ CWRKEPEERP TFEYLQAFLE DYFTSTEPQY QPGENL
c-terminal
v-Src (Rous sarcoma virus oncogene)
...... TFKYLQAQLL PACVLQVAE
pdb: 2src, 1y57

FIGURE 15-12 From SRC to the deregulated kinase v-Src. (a) Schematic representation of two states of SRC. On the left, the SH2 domain folds back onto the phosphotyrosine-530 (latch), thus pushing the SH3 domain against the N-lobe (clamp). The linker region possesses proline-rich sequences, which bind to SH3 and reinforce the interaction with the N-lobe. Dephosphorylation of pY530 and subsequent autophosphorylation of the activation segment (Y419) constitute the activation process. Often, the liberated SH2 domain attaches to an other phosphotyrosine, thus acting as an anchor domain. (b) Structure of SRC in its inactive (left) and active conformations. The different domains are shown in the same color as the amino acids, so as to show the relationship between structure and sequence. The viral oncogene v-Src is similar to SRC, except that it lacks 14 residues in the C-terminal segment. The loss of Y530 renders the protein kinase constitutively active (Wilkerson et al., 1985).

Nonviral oncogenes

Tumors that are not caused by viral infection (such as those caused by chemical carcinogens, radiation, or simply due to the multiple errors that arise as a consequence of aging) also express persistently activated gene products. As an example of the role of oncogenes in cell transformation, mutated forms of Ras are found in 40% of all human cancers (Bos, 1989) and in more than in 90% of pancreatic carcinomas. These oncogenes are gain-of-function mutants of the wild-type proteins. They include receptor tyrosine kinases, adaptor proteins, and guanine nucleotide exchange factors. Serine and threonine protein kinases can also act as oncogenes, but in comparison with tyrosine protein kinases, their contribution is

TABLE 15-3 Components of tyrosine kinase signal transduction cascades manifested as cellular or viral oncogenes

Receptor tyrosine protein kinase	Non-receptor tyrosine protein kinase	Serine/ threonine protein kinase	SH2/SH3 adaptor	Nucleotide exchange factor	GTPase
Bek	Abl	Akt	Crk	Bcr	H-Ras
Eck	Blk	Cot	Nck	Dbl	K-Ras
Elk	Fgr	Mos		Ost	N-Ras
Eph	Fsp	Pim		Tiam	
ErbB	Fyn	Raf		Vav	
Flg	Hck				
Fms	Lck				
Kit	Lyn				
Met	Src				
Neu	Yes				
Ret					
TrkA					
TrkB					
TrkC					

These oncogenes are gain-of-function mutants of the wild-type proteins, such as receptor tyrosine kinases, adaptor proteins, or guanine exchange factors. Serine or threonine protein kinases can also act as oncogenes, but in comparison with tyrosine protein kinases, their contribution is relatively modest.

relatively modest (Spector et al., 1978; Bishop et al., 1978). A number of these mutated proteins operate in the early stages of tyrosine kinase signal transduction pathways. Cells may be transformed as a consequence of hypersecretion of growth factors, expression of variant forms of tyrosine protein kinases (both receptor and non-receptor), overexpression of SH2/SH3-containing adapter proteins, overexpression of serine/threonine protein kinases, or expression of variants of the small GTPases or their accessory proteins. At the downstream end of the signal transduction pathway, variants of transcription factors also act as potent cell transformers (e.g., MYC, FOS). Although tyrosine kinase phosphorylation accounts for only about 5% of total cellular phosphorylation activity, it has a key position in many signal transduction pathways, and it is probably for this reason that the incidence of these genes in malignancy is so high (see Table 15-3).

A good example of cell transformation related to aberrant PTK activity is the role of JAK2 in acute lymphoblastic leukemia (ALL). This is the most common childhood cancer of pre-B-cell origin. These cells rely on paracrine or autocrine cytokine stimulation in order to proliferate and survive. Forms of this disease that are more resistant to chemotherapy express a constitutively activated mutant of JAK2 (Lacronique et al., 1997). Apparently, this serves as a survival signal for these cells making them insensitive to the damage inflicted on DNA by chemotherapy, which would normally induce apoptosis (Meydan et al., 1996). Another example is Bcr–Abl, the cause of CML. This is a fusion protein caused by somatic reciprocal translocation between (paternal) chromosome 9 and (maternal) chromosome 22. The kinase activity of the fusion product, carried by Abl, is deregulated.

ESSAY: NON-RECEPTOR PTKs AND THEIR REGULATION

The non-receptor PTKs comprise a large family that have diverse roles in the control of cell proliferation, differentiation, and death. Some are widely expressed; others are restricted to particular tissues. Their early classification was dominated by the discovery of pp60^{src} in 1960 to the extent that the major group of kinases was simply known as the Src family (Czernilofsky et al., 1980; Smart et al., 1981; Cooper et al., 1986). There are at least 10 known groups of non-receptor PTKs (Figure 15-1 and Table 15-4).

Members of the Src group all share a similar structure. A unique domain at the N-terminus is followed by SH3 and SH2 domains (actually, the very archetypes of these domains that are so widely expressed) followed by a kinase domain and short tail at the C-terminus. Many function by association with macromolecular signaling complexes assembled at membrane sites. Membrane targeting may be promoted by the unique N-terminal domain. Within the Src group, SRC itself (pp60^{c-src}), FYN, LYN, and YES, are myristoylated at the N-terminus. This provides the opportunity for membrane attachment that may be strengthened by palmitoylation at a nearby cysteine. Similarly, members of the TEC family (such as ITK discussed previously) may become membrane associated through their PH domains that can bind polyphosphoinositide lipids (see Chapter 16, “Signaling through the Insulin Receptor”). Other non-receptor PTKs are recruited to their sites of action (and primed for activation) through the association of their SH2 domains with phosphotyrosine residues present in their targets.

Regardless of their location, most Src kinases are held in an inactive state by an intramolecular interaction that links a phosphorylated tyrosine in the C-terminus with the N-terminal SH2 domain (see Figures 15-12 and 15-13) (acting as a latch). Moreover, the SH3 domain makes contacts with the N-lobe (acting as a clamp), and this distorts the coordination of ATP.

TABLE 15-4 Families of non-receptor protein tyrosine kinases

ABL	ACK	CSK	FAK	FES	FRK	JAK	SRC	TEC	SYK
ABL1	TNK1	CSK	PTK2 (FAK)	FER	FRK	JAK1	BLK	BMX	SYK ZAP70
ABL2	TNK2 (ACK1)	MATK	PTK2B (PYK2)	FES	PTK6 (BRK)	JAK2	FGR	BTK	
					SRMS	JAK3	FYN	ITK	
						TYK2	HCK LCK	TEC	
							LYN SRC	TXK	
							YES1		

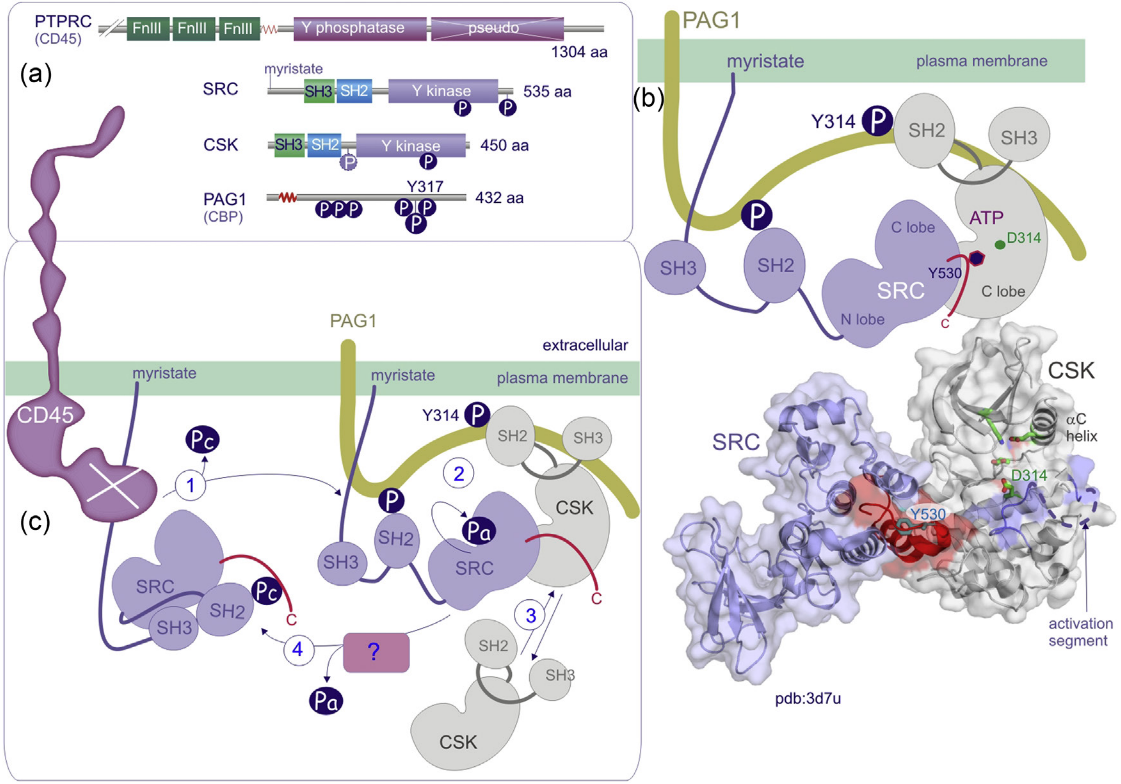


FIGURE 15-13 Regulation of SRC kinase activity by CSK and PTPRC. SRC is inactivated by CSK through tyrosine phosphorylation of its C-terminal tail (1). CSK is recruited in the TRC signaling complex through interaction of its SH2 domain with the transmembrane phosphoprotein PAG1 (formerly CBP). A tyrosine phosphatase removes the phosphate from the tyrosine in the activation segment (2). Inactive SRC can be reactivated by PTPRC (formerly CD45), which removes the phosphate in the C-terminus (Pc) (3). Autophosphorylation of the activation segment completes the job (P_a) (4). This mode of regulation is a good example of how retrocontrol can limit activity. Active SRC eventually phosphorylates PAG1 causing its deactivation by CSK. Loss of Src activity and subsequent dephosphorylation of PAG1 reinitiates a cycle of Src activation. Many tyrosine kinase oncogenes have the effect of obliterating such oscillations because they are unable to adopt their inactive state.

Activation requires removal of the C-terminal phosphate, and then phosphorylation of a tyrosine in the activation segment. Together these changes alter the molecular configuration of the binding sites for ATP and substrate; they render the protein kinase catalytically competent.

The major suppressor of SRC activity is CSK (c-Src kinase), itself a member of the CSK group (together with MATK) (Okada et al., 1991; Okada and Nakagawa, 1988). It suppresses kinase activity through phosphorylation of the aforementioned tyrosine residue located in the C-terminus (Y530 in human SRC). CSK acts as a master switch for all Src members (SRC, FYN, YES, HCK, GFR, BLK, LYN, and LCK). CSK is an unusual PTK because it lacks the conventional mechanisms of regulation. It has no C-terminal tyrosine residue, it is not attached to a myristate, and it does not require phosphorylation of its activation segment. Moreover, it lacks a conventional substrate-binding site due to a small deletion in the activation segment. The kinase is considered to be constitutively active, yet it does not readily phosphorylate substrates. Full activity is only expressed when its SH2 domain attaches to a phosphotyrosine and when it encounters a member of the Src-type kinase, other substrates are more or less ignored. Effectively, the highly conserved C-terminal sequence (aa 505–525) of Src kinases dock at the edge of the active site of CSK, thus bringing the target tyrosine near the catalytic residue (D314) (Okada, 2012; Levinson et al., 2008). Other substrates simply fail to enter the catalytic cleft.

However, how does this cytosolic enzyme interact effectively with the plasma membrane-associated Src kinases? It seems likely that the transmembrane-docking phosphoprotein PAG1 (formerly Cbp) (Kawabuchi et al., 2000) recruits CSK. PAG1 is associated with glycosphingolipid-enriched microdomains 1 (lipid rafts), and one of its phosphotyrosines (Y317) binds the SH2 domain of CSK. Return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases" in particular Figure 10-8, for more information of how SH2 domains recognize phosphotyrosine peptides. As already mentioned, this binding affects the conformation of the catalytic domain, thereby increasing CSK activity toward Src-family kinases (Wong et al., 2005) (see Figure 15-13). The mode of operation described earlier implies that when Src kinases express their activity and phosphorylate substrate, they also attract CSK and signal their own inactivation (Howell and Cooper, 1994).

The inhibitory action of CSK is coupled to the removal of the phosphate from the activation segment of SRC by a phosphatase of unknown identity. Reactivation of SRC might then initially involve the dephosphorylation of PAG1 by a tyrosine phosphatase, among which PTPRC (CD45) is a good candidate. We return to PTPRC, as part of the immunological synapse in which TCR and MHCII operate, in Chapter 18, "Protein Phosphatases," Section "PTPRC and the regulation of immune cell function."

References

Andreotti, A.H., Schwartzberg, P.L., Joseph, R.E., Berg, L.J., 2010. T-cell signaling regulated by the Tec family kinase, Itk. Cold Spring Harb. Perspect. Biol. 2, a002287.

Avots, A., Buttmann, M., Chuvpilo, S., Escher, C., Smola, U., Bannister, A.J., Rapp, U.R., Kouzarides, T., Serfling, E., 1999. CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. Immunity 10, 515–524.

Barber, E.K., Dasgupta, J.D., Schlossman, S.F., Trevillyan, J.M., Rudd, C.E., 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. Proc. Natl. Acad. Sci. U.S.A. 86, 3277–3281.

Bishop, J.M., Baker, B., Fujita, D., McCombe, P., Sheiness, D., Smith, K., Spector, D.H., Stehelin, D., Varmus, H.E., 1978. Genesis of a virus-transforming gene. Natl. Cancer Inst. Monogr. 219–223.

Bos, L., 1989. ras oncogenes in human cancer: a review. Cancer Res. 49, 4682–4689.

Braiman, A., Barda-Saad, M., Sommers, C.L., Samelson, L.E., 2006. Recruitment and activation of PLCgamma1 in T cells: a new insight into old domains. EMBO J. 25, 774–784.

Burg, D.L., Furlong, M.T., Harrison, M.L., Geahlen, R.L., 1994. Interactions of Lyn with the antigen receptor during B cell activation. J. Biol. Chem. 269, 28136–28142.

Cantrell, D.A., 1996. T cell receptor signal transduction pathways. Annu. Rev. Immunol. 14, 259–274.

Chill, J.H., Quadt, S.R., Levy, R., Schreiber, G., Anglister, J., 2003. The human type I interferon receptor: NMR structure reveals the molecular basis of ligand binding. Structure 11, 791–802.

Colombetti, S., Basso, V., Mueller, D.L., Mondino, A., 2006. Prolonged TCR/CD28 engagement drives IL-2-independent T cell clonal expansion through signaling mediated by the mammalian target of rapamycin. J. Immunol. 176, 2730–2738.

Cooper, J.A., Gould, K.L., Cartwright, C.A., Hunter, T., 1986. Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science 231, 1431–1434.

Czernilofsky, A.P., Levison, A.D., Varmus, H.E., Bishop, J.M., Tischer, E., Goodman, H.M., 1980. Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product. Nature 287, 198–203.

De Luca, A., Baldi, A., Esposito, V., Howard, C.M., Bagella, L., Rizzo, P., Caputi, M., Pass, H.I., Giordano, G.G., Baldi, F., Carbone, M., Giordano, A., 1997. The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. Nat. Med. 3, 913–916.

Dierlamm, J., Baens, M., Wlodarska, I., Stefanova-Ouzounova, M., Hernandez, J.M., Hossfeld, D.K., De Wolf-Peeters, C., Hagemeijer, A., Van den Berghe, H., Marynen, P., 1999. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood 93, 3601–3609.

Doherty, J., Freund, R., 1997. Polyomavirus large T antigen overcomes p53-dependent growth arrest. Oncogene 14, 1923–1931.

Dustin, M.L., Tseng, S.Y., Varma, R., Campi, G., 2006. T cell-dendritic cell immunological synapses. Curr. Opin. Immunol. 18, 512–516.

Ellermann, V., Bang, O., 1908. Experimentelle leukämie bei hünern. Zentr Bakteriol. Parasitenk 4, 595–609.

Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S.H., Tanasa, B., Hogan, P.G., Lewis, R.S., Daly, M., Rao, A., 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441, 179–185.

Gacon, G., Fagard, R., Boissel, J.P., Fischer, S., Reibel, L., Piau, J.P., Schapira, G., Comoglio, P.M., 1984. Identification of a 58,000 daltons phosphoprotein with tyrosine protein kinase activity in a murine lymphoma cell line. Biochem. Biophys. Res. Commun. 122, 563–570.

Genot, E., 1994. Interferon α and intracytoplasmic free calcium in hairy cell leukemia cells. Leuk. Lymphoma 12, 373–381.

Gwack, Y., Sharma, S., Nardone, J., Tanasa, B., Iuga, A., Srikanth, S., Okamura, H., Bolton, D., Feske, S., Hogan, P.G., Rao, A., 2006. A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. Nature 441, 646–650.

Haspel, R.L., Darnell, J.E., 1999. A nuclear protein tyrosine phosphatase is required for the inactivation of Stat1. Proc. Natl. Acad. Sci. U.S.A. 96, 10188–10193.

Heit, J.J., Apelqvist, A.A., Gu, X., Winslow, M.M., Neilson, J.R., Crabtree, G.R., Kim, S.K., 2006. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature 443, 345–349.

Hermiston, M.L., Xu, Z., Weiss, A., 2003. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. 21, 107–137.

Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M., Korsmeyer, S.J., 1991. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc. Natl. Acad. Sci. U.S.A. 88, 6961–6965.

Honda, K., Yanai, H., Takaoka, A., Taniguchi, T., 2005. Regulation of the type I IFN induction: a current view. Int. Immunol. 17, 1367–1378.

Howell, B.W., Cooper, J.A., 1994. Csk suppression of Src involves movement of Csk to sites of Src activity. Mol. Cell Biol. 14, 5402–5411.

Ihle, J.N., 1995. Cytokine receptor signalling. Nature 377, 591–594.

Isaacs, A., Lindemann, J., 1957. Virus interference. I. The interferon. Proc. R. Soc. Lond. B Biol. Sci. 147, 258–267.

Kantarjian, H.M., Talpaz, M., O'Brien, S., Jones, D., Giles, F., Garcia-Manero, G., Faderl, S., Ravandi, F., Rios, M.B., Shan, J., Cortes, J., 2006. Survival benefit with imatinib mesylate versus interferon-alpha-based regimens in newly diagnosed chronic-phase chronic myelogenous leukemia. Blood 108, 1835–1840.

Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhowsky, A., Okada, M., 2000. Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature 404, 999–1003.

Kidd, J.G., Beard, J.W., Rous, P., 1936. Serological reactions with a virus causing rabbit papillomas which become cancerous. J. Exp. Med. 64, 63–77.

Kollman, J.M., Merdes, A., Mourey, L., Agard, D.A., 2011. Microtubule nucleation by γ-tubulin complexes. Nat. Rev. Mol. Cell Biol. 12, 709–721.

Kortum, R.L., Rouquette-Jazdanian, A.K., Samelson, L.E., 2013. Ras and extracellular signal-regulated kinase signalling in thymocytes and T cells. Trends Immunol. 34, 259–268.

Lacronique, V., Boureux, A., Valle, V.D., Poirel, H., Quang, C.T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J., Bernard, O.A., 1997. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science 278, 1309–1312.

Lenz, G., Davis, R.E., Ngo, V.N., Lam, L., George, T.C., Wright, G.W., Dave, S.S., Zhao, H., Xu, W., Rosenwald, A., et al., 2008. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science 319, 1676–1679.

Levinson, N., Seeliger, M.A., Cole, P.A., Kuriyan, J., 2008. Structural basis for the recognition of c-Src by its inactivator Csk. Cell 134, 124–134.

Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., Shuai, K., 1998. Inhibition of Stat1-mediated gene activation by PIAS1. Proc. Natl. Acad. Sci. U.S.A. 95, 10626–10631.

Liu, Y.C., Penninger, J., Karin, M., 2005. Immunity by ubiquitylation: a reversible process of modification. Nat. Rev. Immunol. 5, 941–952.

Lucas, P.C., Yonezumi, M., Inohara, N., McAllister-Lucas, L.M., Abazeed, M.E., Chen, F.F., Yamaoka, S., Seto, M., 2001. Bcl10 and MALT1, independent targets of chromosomal translocation in MALT lymphoma, cooperate in a novel NF-kappa B signaling pathway. J. Biol. Chem. 276, 19012–19019.

Lupardus, P.J., Ultsch, M., Wallweber, H., Kohli, B.P., Johnson, A.R., Eigenbrot, C., 2014. Structure of the pseudokinase-kinase domains form protein kinase TYK2 reveals a mechanism for Janus kinase (JAK) autoinhibition. Proc. Natl. Acad. Sci. U.S.A. 111, 8025–8030.

Marth, J.D., Peet, R., Krebs, E.G., Perlmutter, R.M., 1985. A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. Cell 43, 393–404.

Metzger, H., 1992. The receptor with high affinity for IgE. Immunol. Rev. 125, 37–48.

Meydan, N., Grunberger, T., Shahar, M., Arpaia, E., Lapidot, Z., Leeder, J.S., Freedman, M., Cohen, A., Gazit, A., Levitzki, A., Roifman, C.M., 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 379, 645–648.

Min, L., Joseph, R.E., Fulton, D.B., Andreotti, A.H., 2009. Itk tyrosine kinase substrate docking is mediated by a nonclassical SH2 domain surface of PLCγ1. Proc. Natl. Acad. Sci. U.S.A. 106, 21143–21148.

Morris, R.E., 1991. Rapamycin: FK506's fraternal twin or distant cousin? Immunol. Today 12, 137–140.

Myers, M.D., Dragone, L.L., Weiss, A., 2005. Src-like adaptor protein down-regulates T cell receptor (TCR)-CD3 expression by targeting TCRzeta for degradation. J. Cell Biol. 170, 285–294.

Neumeister, E.N., Zhu, Y., Richard, S., Terhorst, C., Chan, A.C., Shaw, A.S., 1995. Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. Mol. Cell Biol. 15, 3171–3178.

Okada, M., Nakagawa, H., 1988. Identification of a novel protein tyrosine kinase that phosphorylates pp60c-src and regulates its activity in neonatal rat brain. Biochem. Biophys. Res. Commun. 154, 796–802.

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., Nakagawa, H., 1991. CSK: a protein-tyrosine kinase involved in regulation of Src family kinases. J. Biol. Chem. 266, 24249–24252.

Okada, M., 2012. Regulation of the Src family kinases by Csk. Intern. J. Biol. Sci. 8, 1385–1397.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J.P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P.G., Rao, A., 2000. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol. Cell 6, 539–550.

Palacios, E.H., Weiss, A., 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 23, 7990–8000.

Pelzer, C., Cabalzar, K., Wolf, A., Gonzalez, M., Lenz, G., Thome, M., 2013. The protease activity of the paracaspase MALT1 is controlled by monoubiquitinylation. Nat. Immunol. 14, 337–345.

Poulin, D.L., DeCaprio, J.A., 2006. Is there a role for SV40 in human cancer? J. Clin. Oncol. 24, 4356–4365.

Qiao, Q., Yang, C., Zheng, C., Fontán, L., David, L., Yu, X., Bracken, C., Rosen, M., Melnick, A., Egelman, E.H., Wu, H., 2013. Structural architecture of the CARMA1/Bcl10/MALT1 signalosome: nucleation-induced filamentous assembly. Mol. Cell 51, 766–779.

Rebeaud, F., Hailfinger, S., Posevitz-Fejfar, A., Tapernoux, M., Moser, R., Rueda, D., Gaide, O., Guzzardi, M., Iancu, E.M., Rufer, N., Fasel, N., Thome, M., 2008. The proteolytic activity of the paracaspase MALT1 is key in T cell activation. Nat. Immunol. 9, 272–281.

Reis e Sousa, C., 2006. Dendritic cells in a mature age. Nat. Rev. Immunol. 6, 476–483.

Robinson, D.R., Wu, Y.M., Lin, S.F., 2000. The protein tyrosine kinase family of the human genome. Oncogene 19, 5548–5557.

Saibil, S.D., Deenick, E.K., Ohashi, P.S., 2007. The sound of silence: modulating anergy in T lymphocytes. Curr. Opin. Immunol. 19, 658–664.

Schutz, G.J., Kada, G., Pastushenko, V.P., Schindler, H., 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. EMBO J. 19, 892–901.

Shuai, K., Liu, B., 2005. Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat. Rev. Immunol. 5, 593–605.

Shual, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z., Darnell, J.E., 1993. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature 366, 580–583.

Simons, K., Ikonen, E., 1997. Functional rafts in cell membranes. Nature 387, 569–572.

Smart, J.E., Oppermann, H., Czernilofsky, A.P., Purchio, A.F., Erikson, R.L., Bishop, J.M., 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src). Proc. Natl. Acad. Sci. U.S.A. 78, 6013–6017.

Spector, D.H., Varmus, H.E., Bishop, J.M., 1978. Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. Proc. Natl. Acad. Sci. U.S.A. 75, 4102–4106.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Sun, L., Deng, L., Ea, C.K., Xia, Z.P., Chen, Z.J., 2004. The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol. Cell 14, 289–301.

Swameye, I., Muller, T.G., Timmer, J., Sandra, O., Klingmuller, U., 2003. Identification of nucleocytoplasmic cycling as a remote sensor in cellular signaling by databased modeling. Proc. Natl. Acad. Sci. U.S.A. 100, 1028–1033.

Syed, R.S., Reid, S.W., Li, C., Cheetham, J.C., Aoki, K.H., Liu, B., Zhan, H., Osslund, T.D., Chirino, A.J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B.A., Matthews, D.J., Wendoloski, J.J., Egrie, J., Stroud, R.M., 1998. Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature 395, 511–516.

Takaoka, A., Yanai, H., 2006. Interferon signalling network in innate defence. Cell Microbiol. 8, 907–922.

Timofeeva, O.A., Plisov, S., Evseev, A.A., Peng, S., Jose-Kampfner, M., Lovvom, H.N., Dome, J.S., Perantoi, A.O., 2006. Serine-phosphorylated STAT1 is a prosurvival factor in Wilms' tumor pathogenesis. Oncogene 25, 7555–7564.

Uren, A.G., O'Rourke, K., Aravind, L., Pisabarro, M.T., Seshagiri, S., Koonin, E.V., Dixit, V.M., 2000. Identification of paracaspases and metacaspases. Two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol. Cell 6, 961–967.

Valentino, L., Pierre, J., 2006. JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem. Pharmacol. 71, 713–721.

van Boxel-Dezaire, A.H., Rani, M.R., Stark, G.R., 2006. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 25, 361–372.

van den Broek, M.F., Muller, U., Huang, S., Aguet, M., Zinkernagel, R.M., 1995. Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. J. Virol. 69, 4792–4796.

Warthin, A.S., 1904. Trans. Assoc. Am. Phys. 19, 421.

Warthin, A.S., 1907. Leukemia of the common fowl. J. Infect. Dis. 4, 369–380.

Weir, M.R., Fink, J.C., 1999. Risk for posttransplant diabetes mellitus with current immunosuppressive medications. Am. J. Kidney Dis. 34, 1–13.

Wilkerson, V.W., Bryant, D.L., Parsons, J.T., 1985. Rous sarcoma virus variants that encode src proteins with an altered carboxy terminus are defective for cellular transformation. J. Virol. 55, 314–321.

Willis, T.G., Jadayel, D.M., Du, M.Q., Peng, H., Perry, A.R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P.G., Dyer, M.J., 1999. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. Cell 96, 35–45.

Wong, L., Lieser, S.A., Miyashita, O., Miller, M., Tasken, K., Onuchic, J.N., et al., 2005. Coupled motions in the SH2 and kinase domains of Csk control Src phosphorylation. J. Mol. Biol. 351, 131–143.

Wu, T.R., Hong, Y.K., Wang, X.D., Ling, M.Y., Dragoi, A.M., Chung, A.S., Campbell, A.G., Han, Z.Y., Feng, G.S., Chin, Y.E., 2002. SHP-2 is a dual-specificity phosphatase involved in Stat1 dephosphorylation at both tyrosine and serine residues in nuclei. J. Biol. Chem. 277, 47572–47580.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R.P., Samelson, L.E., 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 92, 83–92.

Zhong, M., Henriksen, M.A., Takeuchi, K., Schaefer, O., Liu, B., ten, H.J., Ren, Z., Mao, X., Chen, X., Shuai, K., Darnell Jr., J.E., 2005. Implications of an antiparallel dimeric structure of nonphosphorylated STAT1 for the activation-inactivation cycle. Proc. Natl. Acad. Sci. U.S.A. 102, 3966–3971.

Zhou, H., Wertz, I., O'Rourke, K., Ultsch, M., Seshagiri, S., Eby, M., Xiao, W., Dixit, V.M., 2004. Bcl10 activates the NF-kappaB pathway through ubiquitinylation of NEMO. Nature 427, 167–171.

CHAPTER

16

Signaling through the Insulin Receptor: Phosphoinositide 3-Kinases and AKT

Imagine a viva voce examination at one of the more august universities. A student is being questioned about insulin and how it works. After some embarrassed hesitation, he assures the examiners that that he had known how it works, but unfortunately he has now forgotten. "What a pity," comes the response "This means that now, nobody knows."

INSULIN RECEPTOR-SIGNALING: IT TOOK A LITTLE TIME TO WORK OUT THE DETAILS

As should be abundantly clear (return to Chapter 1, "Prologue"), a lack of knowledge about how a particular drug or therapy works has never been an impediment to the doctors working in the clinic. Indeed, for most practitioners, this is generally a very secondary consideration. A well-known case in point is aspirin, first introduced by the Bayer Company in 1898. With the wholehearted encouragement of the medical profession, it has been consumed by the tonne and with good cause too. Yet, it was more than 80 years before its most sensitive target, cyclo-oxygenase, was identified as preventing the synthesis of prostaglandins (Vane, 1971; Roth et al., 1975). Another example is the treatment of childhood (type I) diabetes with insulin (Murray, 1971; Henderson, 1971). Several glycogenolytic hormones (adrenaline, glucagon, vasopressin, and growth hormone) act to mobilize the metabolic stores of carbohydrate and fat, to maintain the concentration of blood glucose, but insulin uniquely has the reverse effect. It increases the net uptake of glucose from the blood into the tissues and increases its conversion to glycogen (and triglyceride), at the same time inhibiting their breakdown. Since the acute effects of the glycogenolytic hormones all require elevations of cyclic AMP or Ca^{2+}, it follows that the actions of insulin must be mediated through a third route, one that escapes

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the attentions of either protein kinase A (cAMP sensitive) or protein kinase C (Ca^{2+}/diacylglycerol sensitive). Only in 1980 did it become apparent that the mechanism involves autophosphorylation of the receptor (Kasuga et al., 1982).The discovery of protein AKT (also known as PKB) and the 3-phosphorylated inositol lipids eventually provided the key.

The association of diabetes mellitus with the pancreas was established by the work of Oscar Minkowski (von Mehring and Minkowski, 1890). A dog, from which he had removed the pancreas, began to suffer an uncontrollable polyuria. The following day, there being no cage large enough to accommodate the animal (which had been well house-trained), it was kept tied up in the laboratory. According to the story (subsequently denied by Minkowski), the assistant noticed that flies settled wherever it had urinated. Regardless of this, the animal passed 12% of sugar and was suffering from diabetes mellitus. It is customary to credit the Canadians, Banting and Best with the discovery of insulin, their colleagues McLeod and Collip somehow standing close by (or not quite so close by in the case of McLeod, who may have spent some of the critical months visiting his ancestors at the Isle of Skye). For sure, were it not for Banting's certainty and indeed obsession, it is unlikely that patients would have been successfully treated within a few months of the commencement of the experimental trials. However, it is legitimate to ask whether the Canadians were actually the first discoverers of insulin. Here, we are not considering the widely canvassed claim of Nicholas Paulescu (Murray, 1971; Henderson, 1971) who was certainly in the race at about the same time but that of Eugene Gley who was working 25 years earlier. Sadly, his right to scientific immortality fails on two counts. First, he was never in a position to apply his preparation in the treatment of patients. This is the importance of Banting and Best's contribution. Whether he had the means to make the repeated and rapid analyses of blood glucose concentration, necessary to monitor its anti-glycosuric effect is uncertain. More likely, he just lacked the audacity to inject his preparation into people, a restraint to which Banting was quite insensitive. Anyway, Gley was working in 1895 and all this had to wait for another quarter century, but we do know from his report that there can be little doubt that he had successfully isolated an anti-glycosuric agent from extracts of pancreas. He provides sufficient detail, rare in his day, for the modern reader to have some confidence that his preparation was as good as he claimed. But where was his report all this time? Instead of going public, Gley sealed it in an envelope which he placed into the hands of the secretary of the Société de Biologie de Paris, with the firm instruction that it was not to be opened until directed by him (Gley, 1922). With this bizarre act he waived all claims to be credited as the discoverer of insulin and so it lay hidden until word came through from Canada in 1921. The application of insulin was certainly one of the key milestones of modern medical practice, yet its mechanism of action as a glucose-lowering hormone has been widely regarded as a "mystery,"

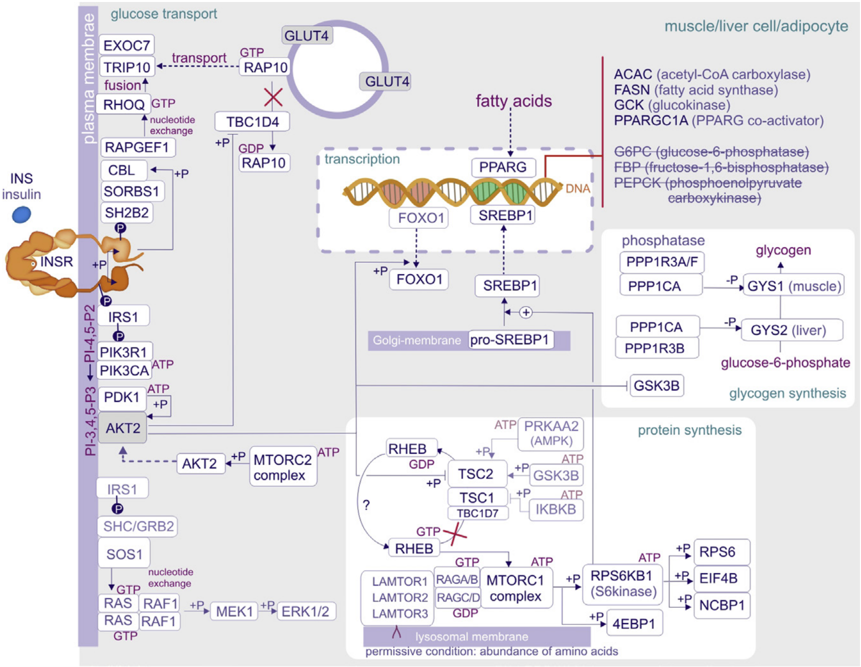


FIGURE 16-1 Overview of insulin receptor signaling pathways. Insulin causes tyrosine phosphorylation of the intracellular segment of its receptor, and this results in the formation of a signaling complex via SH2-mediated recruitment of scaffold proteins and effectors. Four main events follow, translocation of vesicles possessing the glucose transporter GLUT4, activation of the protein synthesis through mTOR, activation of glycogen synthesis through inhibition of GSK3B, and a change in gene transcription (induction of genes that facilitate glucose and fatty acid conversion into glycogen and triglycerides, respectively. Genes that code for components of the glycolysis pathway are temporally inhibited (represented by a strikethrough).

even into the last decade of the twentieth century (Freychet, 1990; Foster and McGarry, 2006).

Insulin has many functions ranging from growth and/or survival factor, stimulation of glucose uptake, stimulation of the production of glycogen in liver and muscle and triglycerides in adipocytes, stimulation of protein synthesis, and induction of a satiety signal in the arcuate nucleus, just to mention few. In this chapter, we outline how it stimulates the uptake and processing of glucose into glycogen and boosts protein synthesis. We also show how the general metabolic state, symbolically represented by the concentration of 5′-AMP, interferes with insulin signaling through the AMPK pathway. We use this particular context to highlight the functioning of PI3-kinase (lipid kinase) and its role in the activation of AKT and, further downstream, of MTOR (protein serine/threonine kinase). An overview of insulin signaling processes discussed here is presented in Figure 16-1.

WEB RESOURCES

- For a more detailed source about the discovery and the discoverers of insulin, with copies of numerous original documents, we suggest a dedicated Website maintained by the University of Toronto. URL: http://link.library.utoronto.ca/insulin/.
- The Kyoto Encyclopedia of Genes and Genomes (KEGG) has an insulin pathway map, entry code "map04910" URL: http://www.genome.jp/.

SIGNALING THROUGH PHOSPHOINOSITIDES

In Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," we discussed phospholipase C, that utilizes the minority phospholipid phosphatidylinositol 4,5 bisphophate (PI-4,5,-P2) as its substrate. Cleavage, by phospholipase C, of the phospholipid headgroup produces the soluble second messenger inositol trisphosphate (IP_3), which releases Ca^{2+} from intracellular stores into the cytosol. The other product is the membrane lipid diacyclglycerol which activates protein kinase C (PRKC family). PI-4,5,-P2 was found in "resting cells" (not treated with any particular agent) and its level in cell outer membranes was originally considered to be under homeostatic control; it was therefore not classed as a signaling molecule. However, this is oversimplification. Quite apart from being a substrate for phospholipase C, the head group of inositol lipids provides an important anchoring point for cytosolic proteins, principally those which possess a PH, PX, FYVE domain, or a specialized-WD repeat (Figures 16-2 and 16-3). Furthermore, this phospholipid also exists in other cellular membranes, such as those of the Golgi apparatus and transport vesicles that communicate with it (endo/exo cytosis). Within the plasma membrane it is distributed unevenly, tending to segregate into dynamic microdomains also rich in cholesterol and sphingolipids, commonly referred to as lipid rafts (briefly discussed in Chapter 15, "Activating the Adaptive Immune System"). Exactly how levels of PI-4,5-bisphosphate in these lipid rafts are regulated is unclear, but there is evidence for local control through the phosphatidylinositol phosphate kinases that convert phosphatidylinositol-4-phosphate to phosphatidyl-4,5-bisphosphate. These phosphatidylinositol kinases are under control of monomeric GTPases such as RHOA (Chong et al., 1994) and ARF6 (Honda et al., 1999).

Although it is far from clear that PI-4,5-P_2 qualifies as a signaling molecule, inositol lipids that are phosphorylated in the 3-position certainly do. These are products of the PI3-kinases that phosphorylate

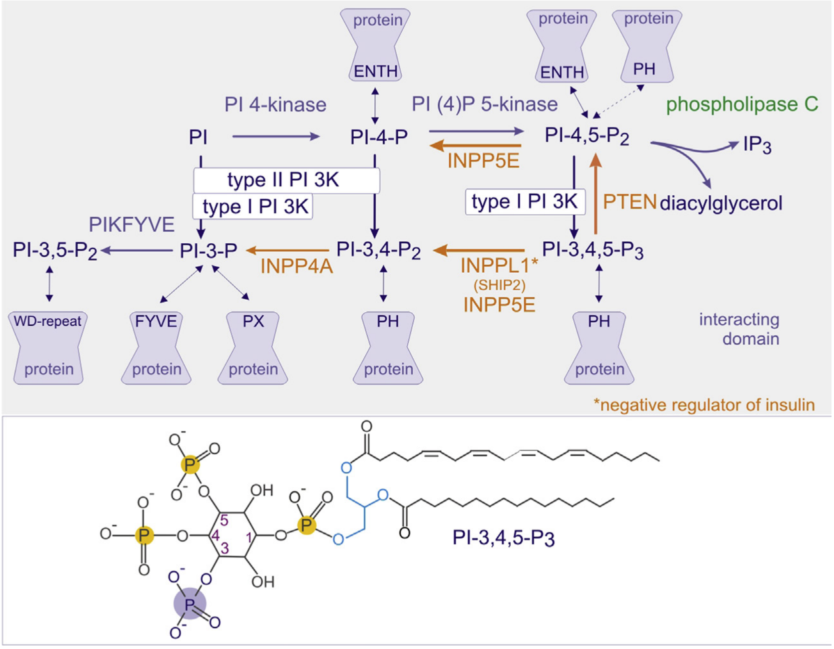
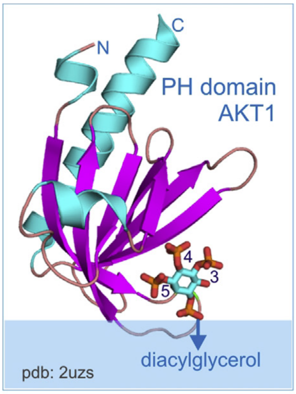


FIGURE 16-2 Phosphoinositide 3-kinases and the generation of 3-phosphorylated lipids. The PI3-kinases phosphorylate the inositol lipids in the 3-position. 3-Phosphorylated inositol lipids are not substrates for phospholipase. The phosphatases PTEN and SHIP2 play an essential role in removing PI-3,4,5 either by removing the 3-phosphate or the 5-phosphate. INPP5A removes the 5-phosphate from 3,4,5 and 4,5 phosphorylated inositols. INPP4A removes the 4-phosphate. PI-3-P is the substrate of PIKFYVE kinase, giving rise to PI-3,5-P2. Different protein domains recognize the phosphoinositide lipids and these play a role in protein recruitment to the membrane. The lower panel shows the composition of phosphatidylinositol-3,4,5-trisphosphate.

phosphatidylinositol and its derivatives (PI-4-P and PI-4,5-P_2) to give PI-3-P, PI-3,4-P_2 or PI-3,4,5-P_3 (Figure 16-2).

PHOSPHATIDYL INOSITOL 3-KINASE

It first caught attention as being a protein associated with the polyoma middle T antigen (Whitman et al., 1985). The potent transforming activity of middle T antigen is dependent on its association with a number of cellular proteins such as members of the Src family of protein kinase and the newly identified phosphatidylinositol kinase (Talmage et al., 1989). It was then shown that the novel phosphatidylinositol kinase had the unprecedented ability to phosphorylate phosphoinositides on the 3′ position of the inositol ring. In the case the enzyme was activated by platelet-derived growth factor in intact cells, it showed a special preference for PI-4,5-P2 (Whitman et al., 1988; Auger et al., 1989). At the same time, PI-3,4,5-P_3 was also detected



protein	PH specificity
ADRBK1	PI-4,5-P$_2$, PI-3,4,5-P$_3$
AKT	PI-3,4-P$_2$, PI-3,4,5-P$_3$
BTK	PI-3,4,5-P$_3$
CYTH3	PI-3,4,5-P$_3$
PLCG1	PI-4,5-P$_2$
PDK1	PI-3,4-P$_2$, PI-3,4,5-P$_3$
PLEK (pleckstrin)	PI-3,4-P$_2$
PLEKHA1	PI-3,4-P$_2$
SYNGAP1	PI-3,4,5-P$_3$

FYVE	
EEA1	PI-3-P
HGS	PI-3-P
PIKFYVE	PI-3-P
SAR1A	PI-3-P

WD-repeat	
WIPI1	PI-3,5-P$_2$
WIPI2	PI-3,5-P$_2$

PX	
NCF1 (p47phox)	PI-3,4-P$_2$
NCF4 (P40phox)	PI-3-P
SNX1 (nexin)	PI-3-P, PI-3,5-P$_2$
SNX2	PI-3-P

ENTH	
CLINT1	PI-4-P
EPN1	PI-4,5-P$_2$
SNAP91	PI-4,5-P$_2$

FIGURE 16-3 Phosphoinositide-binding domains, some proteins that possess them, and the specificity of the phosphoinositides they bind to.

in human neutrophils after stimulation with formylmethionyl peptides (Traynor Kaplan et al., 1988). Although initially contemplated as a new substrate for phospholipase, and thus the source of a potential new water-soluble second messenger (inositol-1,3,4,5-tetrakis phosphate or IP$_4$), it soon became clear that the intact lipid is a signaling entity. Because its head group offers such a multiplicity of phosphorylation patterns, phosphatidylinositol occupies a special place among phospholipids. Although inositol lipids have been detected in the analyses of bacteria, there is no indication that any of them plays a regulatory role. However, it is clear that some bacteria rely on the inositide metabolism of host cells to regulate their invasiveness (Ireton et al., 1996). Phosphatidyl inositol is present in all eukaryotic cells, though its metabolism in unicellular organisms such as yeast is restricted, since they lack the means to generate either PI-4-P or PI-4,5-P$_2$. The substrate specificity of the yeast PI3-kinase (coded by the gene VPS34) is appropriately limited to PI and therefore it only generates the monophosphorylated derivative PI-3-P (which can then be converted to PI-3,5-P$_2$, essential for Golgi membrane recycling (Gary et al., 1998; Herman et al., 1991)).

Unlike the more familiar PI-4,5-bisphosphate (PIP2), the 3-inositol phospholipids having a phosphate group at 3-position do not serve directly as substrates for phospholipase-C. Instead, they are metabolized by the hydrolysis of the phosphate groups at 3- and 5-positions by the phosphatases PTEN and SHIP2, respectively (Figure 16-2). Both the 3-kinases and the 3-phosphatases are regulated by receptor-mediated processes and it is apparent that the 3-phosphorylated inositol phospholipids are full-fledged second messengers. The difficulty has been in assigning the processes that they regulate.

A family of PI3-kinases

Cloning and screening strategies have revealed a number of enzymes having 3-phosphorylating activity. They comprise a family subdivided into three types that have distinct substrates and various forms of regulation (Figure 16-4). They all have four homologous regions, the kinase domain being the most conserved (Walker et al., 1999).

Class I PI3-kinases

This class of PI3-kinases phosphorylate generate the PtdIns-3,4,5-P3 lipid. They are heterodimers in vivo comprising a regulatory subunit (of various sizes) that maintains the enzyme in a low-activity state and a catalytic subunit (p110). Each exists in various forms. Their multidomain structure (in particular p85) enables them to interact with other signaling proteins allowing activation from different directions (Figure 16-5) (Yu et al., 1998). The SH2 domains enable them to bind to phosphotyrosine residues. Similarly, the SH3 domains allow interaction with proline-rich sequences, present for instance in the focal adhesion kinase TPK2 (formerly FAK), the adaptor molecule SHC, the GTPase-activating protein

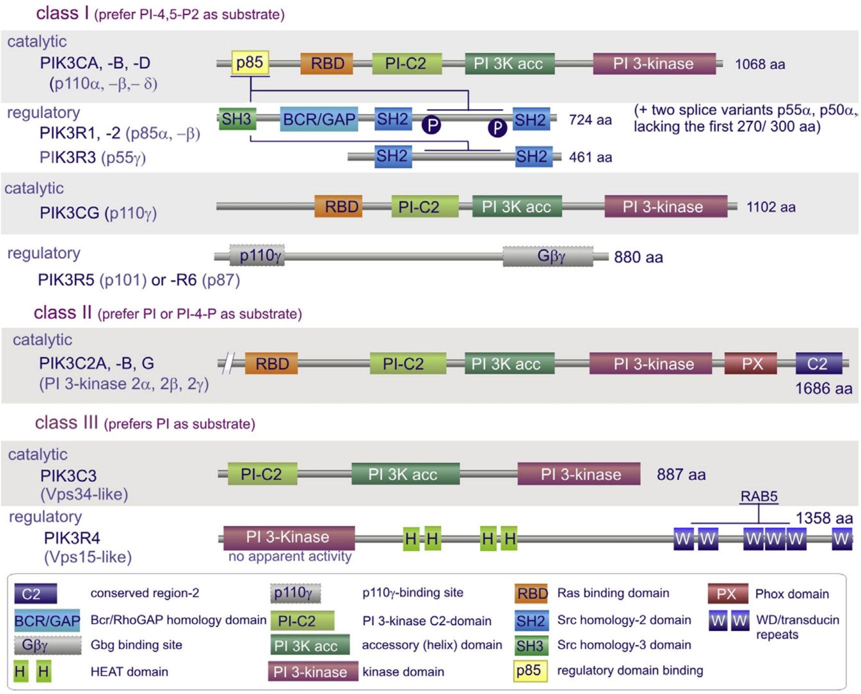


FIGURE 16-4 Classification of phosphoinositide 3-kinases. The enzymes are classified into three classes based on the domains associated with the protein kinase. *Information from Vanhaesebroeck et al. (2010).*

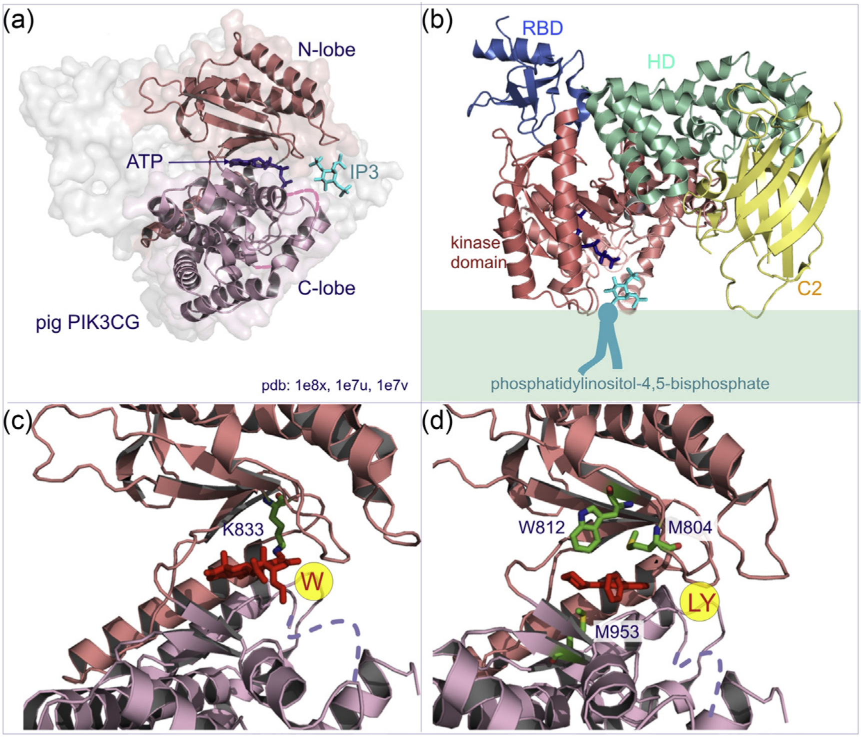


FIGURE 16-5 Structure of PI3-kinase-γ (PIK3CG) and binding sites of the inhibitors wortmannin and LY294002. (a) The catalytic domain of PI3-kinase resembles the protein kinases. It is composed of an N-lobe and a C-lobe and between them the catalytic cleft that harbors ATP and forms the substrate entry point. The activation segment (dotted pink line) determines the affinity of the kinase for phosphatidylinositol lipids (rather than amino acids). (b) PI3-kinase comprises a Ras-binding domain (RBD) (blue), a C2 domain (yellow), a helix domain (green), and a catalytic domain (red). The RBD contacts the N-lobe and to a lesser degree the C-lobe and this suggests that RAS binding will have an allosteric (stimulatory) effect on the catalytic domain. The C2 domain participates in membrane interaction (in analogy with the C2 domain in protein kinase C). The helical domain is common to the PI3-kinase and PI4-kinase families and serves as a spine on which the other domains are fastened. (c) The PI3-kinase inhibitor wortmannin binds covalently to K833, a conserved residue that normally is involved in the coordination of the α-phosphate of ATP. (d) LY294002 also occupies the ATP-binding pocket. Its predominant sites of interaction are offered by M904, Y812, and M953 (hydrophobic/pi-stacking contacts). The view is limited to the catalytic cleft.

ARHGAP1 (formerly Cdc42GAP), or the regulator of TCR signaling, CBL (Rudd and Schneider, 2000). In addition, the p85 subunits contain a BCR/GAP homology domain (Musacchio et al., 1996) that interacts with RAC1 and CDC42, members of the Rho family of GTPases, providing yet further opportunities for regulation (Diekmann et al., 1991).

There are four isoforms of the p110 catalytic subunit (α, β, δ, and γ) all of which contain a kinase domain and an Ras-binding site (RBD)

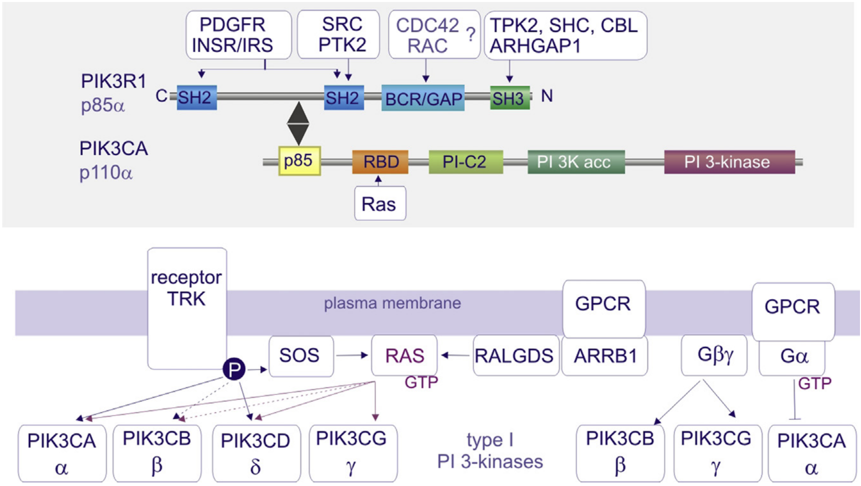


FIGURE 16-6 Multiple pathways to control class I PI3-kinase. Several proteins interact with the p85-regulatory subunit of class 1 PI3-kinases. The receptors for PDGF and insulin or insulin-receptor substrate (IRS) and also the cytoplasmic tyrosine kinases SRC and PTK2 interact at the SH2 domains. A proline-rich region present in PTK2, SHC, CBL, and ARHGAP1 interacts with the SH3 domain. The catalytic subunit p110α can interact directly with RAS. In vitro studies have shown that Rho-related GTPases such as RAC1 and CDC42 interact at the BCR/GAP domain. Through these interactions both receptor tyrosine protein-kinase and GPCR receptors are able to stimulate the class I family of PI3-kinases. In the case of tyrosine kinase-operating receptors, this occurs directly through recruitment via the SH2 domain of the regulatory subunits and indirectly through activation of RAS (via the arrestin and RALGDS pathway). GPCR receptors also have a direct mode of activation; the Gβγ subunits bind at the regulatory subunit PIK3R5 (connected with PIK3CG) and directly bind to PIK3CB. Finally, Gα inhibits PIK3CA.

(Rodriguez-Viciana et al., 1994). In addition, the α-, β-, and δ-isoforms possess interaction sites for the p85 subunit and this causes their recruitment to receptors that operate through tyrosine protein kinases (EGFR, PDGFR, INSR, etc.). These three isoforms are operational in the activation of AKT. The p110γ (PIK3CG) is different, it lacks a p85-binding site but instead associates with a regulatory subunit (PIK3R5 or -R6) that interacts with the βγ subunits of heterotrimeric G proteins (Voigt et al., 2005; Wymann and Pirola, 1998). The class I PI3-kinase enzymes respond to different upstream signals and have different functions. This is apparent in macrophages in which distinct isotypes modulate separate cellular responses. Here, mitogenic signaling induced by colony-stimulating factor-1 is mediated by p110α, whereas actin organization and cell migration require the β- or δ-isoforms (Van Haesebroeck et al., 1999). In mammalian cells, the class I PI3-kinases have roles in the modification of intracellular membranes, affecting the recruitment of the fission and fusion machinery necessary for intracellular vesicle traffic. The class I PI3-kinases are also involved in the formation of phagosomes (Foster et al., 2003). The structure of type I PI3-kinase (pig PIK3CG) is shown in Figure 16-6(a) and (b).

Class II PI3-kinases

The three members of this group, 2α, 2β, and 2γ, have substrate specificity for PI and PI(4)P. They are all monomeric and possess a C-terminal C2 domain. Their mode of activation is unclear as is their role in cellular functioning.

Class III PI3-kinase

This group is represented by PIK3C3, the human homologue of the yeast gene product Vps34. It associates with PIK3R4 and only phosphorylates PI to form PI-3-P. Under conditions of starvation, the activity of PIK3C3 is inhibited, causing restriction of the synthesis of enzymes involved in a pathway that acts via the kinase complex mTORC1 (see below). How PIK3C3 regulates mTORC1 activity is not known but it is clear that it is a key component of the nutrient-sensing system. In yeast, hVsp34 is involved in autophagy, a rescue mechanism that allows renewal of cellular components in times of prolonged starvation (Dann and Thomas, 2006).The mammalian homologue too not only plays a key role in initiation and maturation of autophagosomes but is also instrumental in the transport of lysosomal enzyme precursors to lysosomes and in the transport from early to late endosomes. It is required for the abscission step in cytokinesis (Vanhaesebroeck et al., 2010).

STUDYING THE ROLE OF PI3-KINASE WITH INHIBITORS

It might be thought that the availability of an inhibitor of the PI3-kinases, in this case wortmannin, would have provided an unambiguous key to the understanding of the pathways and cellular functions that they control. This antifungal antibiotic, isolated from *Penicillium wortmannii,* was originally identified as a toxic agent causing acute necrosis of lymphoid tissues, severe myocardial hemorrhage and hemoglobinuria (Gunther et al., 1989). At an appropriate dose, it is also a powerful anti-inflammatory agent (Closse et al., 1981).

At concentrations above 10^{-9} M, wortmannin associates covalently with the p85-regulatory subunit of PI3-kinase (Wymann et al., 1996). Used judiciously, and in parallel with other inhibitors (e.g., Lilly compound LY294002) and independent approaches, wortmannin certainly has its place in the battery of techniques for investigating signal transduction. In the quest for inhibitory effects, however, it has often been applied at high concentrations, leading to false-positive reports of inhibition. Furthermore, due to the existence of multiple isoforms of PI3-kinase, not all of which are targets of this compound, a negative result does not necessarily rule out a role for the products of 3-phosphorylation. The binding sites of wortmannin and LY294002 are shown in Figure 16-6.

PATHWAYS OF ACTIVATION FOR PI3-KINASE

The process by which receptors for growth factors activate the type I PI3-kinases through interaction with the SH2 domain of the p85-regulatory subunits is well established (see Figure 16-5). PI3-kinase can also be recruited and activated by non-receptor tyrosine kinases such as those of the Src family, through the same mechanism, as long as they are located at the membrane (otherwise activity cannot be expressed by lack of substrate). In platelets that have been stimulated by thrombin, it appears that activation of PI3-kinase is linked to TPK2 (focal adhesion kinase). This kinase, associated with integrin signaling and cytoskeletal organization at focal adhesion sites, contains proline-rich regions that interact with the SH3 domain on p85 (Guinebault et al., 1995). In addition, phosphorylation of TPK2 allows interaction with the SH2 domain of p85 and this appears to be the route of activation following attachment of cells to solid substrates. PI3-kinase is also activated by RAS which interacts directly with the catalytic p110α subunit (Rodriguez-Viciana et al., 1994). Other GTPases, particularly those of the Rho family are also involved as regulators (and downfield effectors) in pathways regulated by PI3-kinase (Wymann and Pirola, 1998).

Unfortunately, it has all too readily been assumed that the actions of wortmannin are specific to the 3-kinases, even though it was in use as an inhibitor of myosin light chain kinase for at least a couple of years before its first application as an inhibitor of PI3-kinases (Nakanishi et al., 1992). At concentrations above 10^{-7}M, wortmannin also inhibits a form of PI4-kinase and then, in the micromolar range, in addition to its effects on myosin light chain kinase it possibly inhibits other protein kinases as well. As a result of its uncritical use, the 3-phosphorylated lipids were considered to be implicated in several processes that are quite innocent of any such relationship.

This could be taken as an object lesson in the use of inhibitors in general. Experience advises that the terms "potent" and "highly specific," frequently used to promote pharmacological agents on their first outing, may wear a bit thin after a year or two. As the evidence of side effects accumulates, new "potent" and "highly specific" agents come to take their place. The application of "potent" and "highly specific" inhibitors of calmodulin and later of protein kinase C muddled progress in an earlier generation.

AKT AND ACTIVATION THROUGH PI-3,4,5-P3

The viral oncogene v-akt (acutely transforming retrovirus) encodes a fusion product of a cellular serine/threonine kinase and the viral structural component Gag. This kinase, named AKT, is similar to both PRKCE (PKCε) (73% identity to the catalytic domain) and PRKCA (PKA) (68%), hence its second name PKB (in between A and C). Unlike the other kinases, it contains a PH domain that enables it to bind to polyphosphoinositide head groups. There are three subtypes, all of which show a broad tissue distribution (Figure 16-7). With respect to their structural organization and mode of activation, they belong to the AGC subfamily of serine/threonine protein kinases (see text box).

> Both terms, PKB and AKT, turn up in the literature. AKT1, -2, and -3 are the same as PKBα, -β, and -γ. In the HUGO gene nomenclature database, they are named AKT1–3, the name we employ throughout this book, and they classify among the "pleckstrin homology-domain containing" proteins. In the UniProt database, they are members of the AGC kinase family and they belong to the "RAC" subfamily of serine/threonine protein kinases because the mammalian sequence was discovered as a kinase related to A-kinase (PKA) and C-kinase (PKC) (Jones et al., 1991).

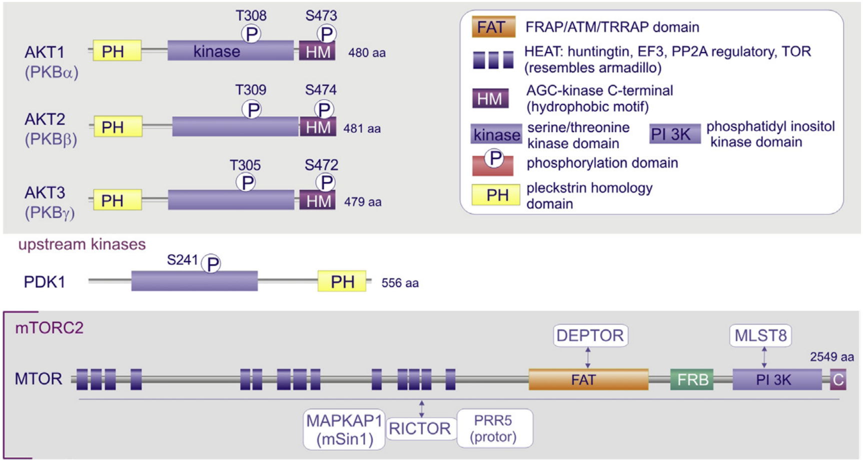


FIGURE 16-7 Classification of the protein kinases AKT. Originally identified as the oncogene in the transforming retrovirus Akt8, there are multiple isoforms of AKT (1, 2, and 3). They all contain a PH domain and an AGC-kinase C-terminal domain harboring the hydrophobic motif (HM) but differ slightly in the locations of their regulatory phosphorylation sites. Activation occurs through phosphorylation by two upstream protein kinases, PDK1 and MTOR. The latter is part of a big complex, named mTORC2 and comprising MAPKAP1, RICTOR, PPR5, DEPTOR, and MLST8.

Shortly after its discovery, it was found that AKT is activated by PI-3,4,5-P3 but the actual mechanism of activation turns out to be far from simple. The phospholipid plays two distinct roles—acting both as a recruiting sergeant and as an activating signal. The first is direct, the lipid head group binding to the PH domain in the N-terminal segment of AKT (Bellacosa et al., 1991) and the other indirect, through recruitment of PDK1.

Before we return to what happens at the membrane, it is important to understand that the three forms of AKT all require phosphorylation at the C-terminal tail, in the hydrophobic segment, and at the activation segment, in order to be catalytically competent (see Figure 16-7 for the residues concerned and Figure 16-8 for the conformational changes induced by phosphorylation) (Yang et al., 2002). Phosphorylation at the hydrophobic segment in the C-terminal tail causes its attachment to the N-lobe, and this brings about a set of positional changes in the αC-helix and in conserved amino acids that coordinate ATP (see Figure 16-8(b)).

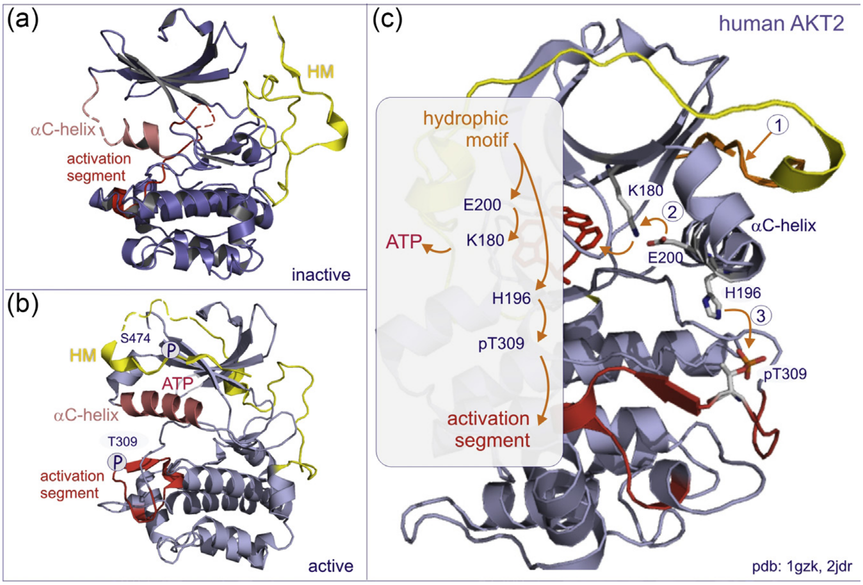


FIGURE 16-8 Structure of inactive and active AKT. (a) In the inactive PKB structure, the various regions of the kinase domains comprising the αC-helix of the N-lobe and the activation segment are disordered. Substrate and ATP do not bind. The organization of the C-terminal segment with its hydrophobic motif (HM, marked yellow) is indicated approximately. (b) Binding of the C-terminal hydrophobic motif (HM, yellow) with the αC-helix is facilitated by phosphorylation of S474. This induces reorganization of the αC-helix (pink) and a second phosphorylation in the activation segment (T309) organizes the activation segment (red). Binding of ATP and substrate ensues. (c). Binding of the hydrophobic motif to the αC-helix (1) leads to structural rearrangements in which E200 engages and correctly positions K180 (2) that coordinates the binding of ATP in the catalytic cleft. The H196 of the ordered αC-helix also engages the PDK1-phosphorylated T309, resulting in a reorientation of the activation segment (3). The kinase now binds both ATP and substrate and is catalytically competent.

Phosphorylation of the hydrophobic segment occurs at birth, on the ribosome, through the action of the mTORC2 complex. This complex contains the serine/threonine protein kinase mTOR surrounded by four regulatory subunits, RICTOR, MLST8, SIN1, and PROTOR (Russell et al., 2011). mTORC2-mediated phosphorylation primes the protein kinase but does not render it catalytically competent because of an unstructured activation segment. For this, it requires phosphorylation by PDK1.

PDK1 too occurs in a primed state but here it is the activation segment that is phosphorylated and ready for action. What is lacking is the correct conformation of N-terminal lobe but, quite unfortunately, PDK1 lacks the required C-terminal tail. To solve the problem it can borrow the hydrophobic segment from AKT but this requires a close encounter. Binding of both kinases to PI-3,4,5-P_3 enables PDK1 and AKT to embrace, an encounter that, as shown below, proves beneficial to both.

Membrane recruitment of AKT reorients the PH domain away from the catalytic cleft so that the activation segment becomes accessible for phosphorylation (Calleja et al., 2007). Once attached to the membrane, the C-terminal tail of AKT swings over to the N-lobe of PDK1 and activates the fellow kinase. In return, PDK1 phosphorylates the activation segment of AKT. When the two separate, PDK1 will return to its primed but catalytically incompetent state (seeking out other members of the AGC-kinases) (Mora et al., 2004), and AKT will remain catalytically competent for some time (until a phosphatase removes the phosphate from the activation segment; the sequence of events is illustrated in Figure 16-9). AKT phosphorylates substrate at the membrane, in the cytosol and perhaps even in the nucleus. Membrane anchoring is an essential step not only in activation but also in subsequent signaling processes because a mutation in the PH domain where a lysine replaces a glutamate (E17K) causes leukemia in mice. This mutation strengthens the electrostatic interactions with the inositol-phosphate head group and keeps AKT1 much longer at the membrane (Carpten et al., 2007). On that same note, the viral oncogene product, v-Akt, has a lipid anchor (myristoyl group). Prolonged attachment may be oncogenic because it facilitates activation or prevents deactivation by phosphatases. It may also prevent phosphorylation events that ensure negative feedback, thus perpetuating the signal.

The AGC family of protein kinases comprises serine/threonine kinases having structural homology with PKA, PKG, and PKC, hence the name AGC. This classification is not solely based on sequence homology but also on activation mechanisms. They contain a C-terminal hydrophobic motif (FxxF[S/T]Y) (except for PDK1). They also share a mode of activation in which (for most of them) PDK1 acts as the master switch. Examples are members of the S6-kinase subfamily (RPS6KB1, RPS6KA3, and RPS6AK5), members of

continued

protein kinase C family (PRKACA, -B, -G, -D, -E, -Q, -H, -Z, and PKN), serum glucocorticoid-responsive kinase (SGK1), and of course AKT family members (Frodin et al., 2002). For the domain architecture of these protein kinases, see Figure 16-9(b). For a comprehensive review, see Pearce et al. (2010).

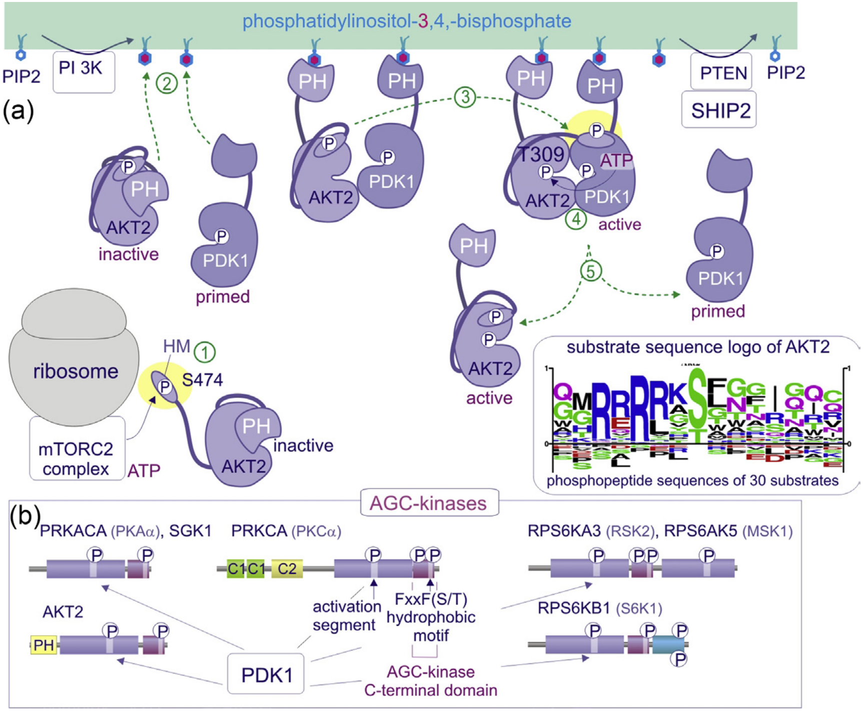


FIGURE 16-9 Activation mechanism of AKT. (a) AKT2 is phosphorylated in the hydrophobic motif of the C-terminal tail on the ribosome. The kinase is primed but not catalytically competent (1). Upon production of PI3,4,5-both AKT2 and PDK1 attach to the membrane with their PH domains (2), which in the case of AKT2 reorients away from the catalytic cleft (thus exposing the activation segment). The phosphorylated hydrophobic motif of AKT2 next binds to the N-lobe of PDK1 causing a rearrangement that renders the kinase fully competent (the activation segment is already phosphorylated) (3). In return PKD1 phosphorylates AKT2 in its activation segment (4). Full kinase activity is achieved once AKT2 has recovered its own C-terminal tail. Detachment of activated AKT2 and primed PDK1 may occur after dephosphorylation of PI-3,4,5-phosphate by PTEN or SHIP2 (5). AKT2 remains active for some time until a phosphatase has removed the phosphate in the activation segment (not shown). (b) Domain architecture of the AGC kinases. The activities are controlled by paired phosphorylation sites, one in a hydrophobic motif, sometimes part of a larger autoregulatory domain, the other in the activation segment. For these kinases, PDK1 acts as the master switch by phosphorylation of the activation segment. The insert shows the phosphopeptide sequence as obtained from 30 substrates of AKT2. *Information from* www.phosphosite.org *and Hornbeck et al. (2004).*

INSULIN: THE ROLE OF IRS, PI3-KINASE AND AKT IN THE REGULATION OF GLYCOGEN SYNTHESIS

From the insulin receptor to AKT

Insulin signaling pathways all commence at the insulin receptor, INSR. The product of a single gene, this is posttranslationally cleaved into an extracellular α-subunit (containing a ligand-binding site) and a transmembrane β-subunit (which has a tyrosine protein-kinase catalytic domain). The mature functional receptor comprises four components, 2α, and 2β, held together by disulfide bonds (Figure 16-10).

In common with the related insulin-like growth factor receptors (IGF1R) and insulin-related orphan receptor (INSRR), the insulin receptor is dimeric in its inactive state. In this respect, it differs from all other tyrosine kinase-containing receptors. Binding of the ligand, insulin, or IGF1, must alter the receptor conformation or the relative position of the receptor subunits in order to allow the intracellular catalytic domains

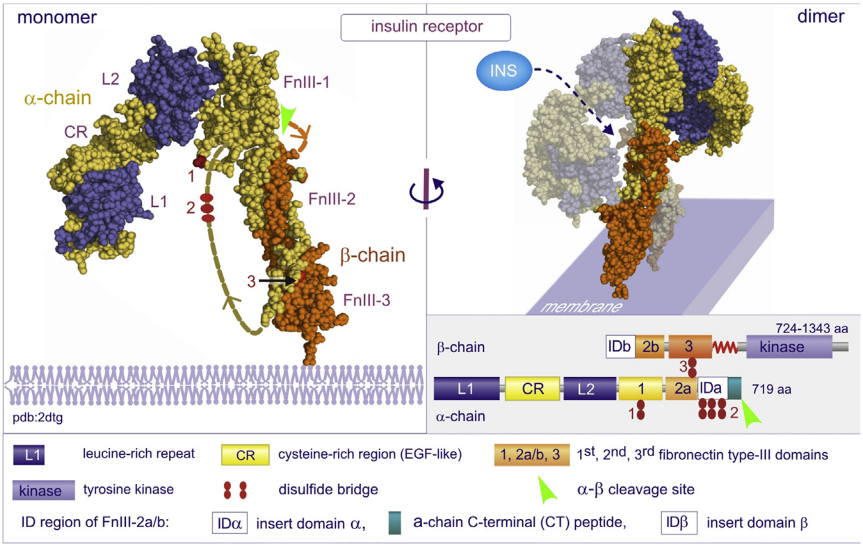


FIGURE 16-10 Insulin receptor structure. The insulin receptor is a homodimer. In the mature form, each component is present as two chains, α and β. The protein is organized in seven domains, L1 (leucine rich region-1), CR (cysteine rich region), L2, FnIII-1 (fibronectin-III-like domain 1), FnIII-2, FNIII-3, and the intracellular tyrosine kinase domain. Both the α and β chains contribute to the FnIII-2 domain. The long insert region (shown as a dotted line) separating FnIII-2a from -2b contains three disulfide bridges (position 2) that link the monomers. FNIII-1 provides a fourth disulfide bridge (position 1). A possible orientation between the two peptides is indicated in the right hand panel (dimer). The insulin-binding space is between the central β-sheet of L1 and the bottom loops of the FnIII-1 domain. *From McKern et al. (2006).*

to approach each other. Three trans-phosphorylations in each activation segment ensue, in orderly sequence, all of which are required for maximal activation (White et al., 1988). Further phosphorylations then occur in positions beyond the catalytic site (see Figure 16-11).

A second peculiarity of the insulin receptor is that it signals through the intervention of large docking proteins. These insulin receptor substrates (IRS-1 to 4) all possess PH and PTB domains. The PTB domains bind directly to the tyrosine phosphorylated region of the receptor which corresponds to pY999 (in the peptide sequence EpYLSAS) and results in phosphorylation of the docking protein. Of the four docking proteins, IRS-1 and -2 are essential for insulin signaling: lack of IRS1 is linked to insulin resistance in muscle and adipose tissue. In mice, absence of IRS2 causes insulin resistance in the liver (among many other developmental defects).

The p85-regulatory subunit of PI3-kinase-α (class I) binds phosphorylated IRS1 or -2 through its SH2 domain (Valverde et al., 1998) (Figure 16-11) so negating the inhibitory constraint imposed on the catalytic subunit and bringing the kinase into the vicinity of its substrate. Prolonged activation of PI3-kinase and the production of 3-phosphorylated polyphosphoinositides encourages a number of serine/threonine kinases to associate with the plasma membrane. Important among these is AKT.

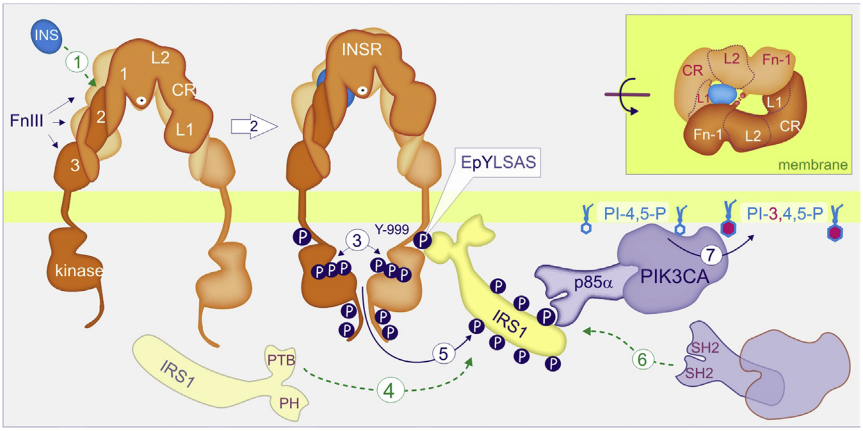


FIGURE 16-11 Activation of PI3-kinase by the insulin receptor. Insulin binding to the receptor dimer (1) induces a conformational change (2) that causes trans-phosphorylation of the activation segments at three tyrosine residues (3). Further phosphorylation follows, both upstream and downstream of the catalytic domain. The IRS1 binds pY999 with its PTB domain (4). Phosphorylation on further tyrosine residues follows (5) generating a docking site for the SH2 domains of the p85-regulatory subunit and activation of PI3-kinase (PIK3CA) (6).

The intervention of PI3-kinase in the activation of AKT is well supported by a variety of observations (Franke et al., 1995). It is quiescent in serum-starved fibroblasts but becomes active shortly after the addition of insulin or platelet-derived growth factor (PDGF). This fails in cells that contain a mutant form of AKT lacking the PH domain and it depends on the presence of phosphotyrosines on IRS1 or on the PDGF receptor (Franke et al., 1997). Of the three members of the AKT family, AKT2 plays the major role; the other two seem not to be involved in insulin signaling (Jiang et al., 2003). Similar to insulin, PDGF is able to activate AKT but is without immediate effect on glucose metabolism. Other factors must be in place to give direction to the signal transduction pathways downstream of these receptors. For instance, the proximity of phosphorylated IRS1 to the insulin receptor relays the activation of AKT in the sense of glucose metabolism. This connection is absent in the case of the PDGF receptor.

Lastly, note that the insulin receptor does not signal exclusively through the IRS because it can also bind the adaptor SH2B2 (formerly APS). This regulates membrane expression of the glucose transporter SCL2A4 (glucose transporter-4) to which we return later on (Hu et al., 2006). Finally, p85α (PIK3R1) can also bind directly to the C-terminal end of INSR.

A point mutation in AKT2 in a patient with severe insulin resistance. A missense point mutation present in the kinase domain of AKT2 is present in members of a family showing autosomal dominant inheritance of severe insulin resistance. This finding provides additional evidence for the essential role for AKT2 in insulin signal transduction (Wilson et al., 2004).

From AKT to glycogen synthase

The minimum sequence motif required for efficient phosphorylation of small peptides by AKT is RxRxx(S/T)(F/L) and there are several substrates in which this is present. With respect to insulin signaling, one such is glycogen synthase kinase-3, GSK3B (GSK3β). It has two unusual features. First, the activation segment is fully structured when dephosphorylated so that it needs no further modification to become active. Second, it recognizes only those substrates that offer an array of serine phosphorylation sites, of which the most C-terminal is already phosphorylated ("primed substrate"). (We considered a similar situation in relation to the phosphorylation of β-catenin; return to Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation," Section "WTN

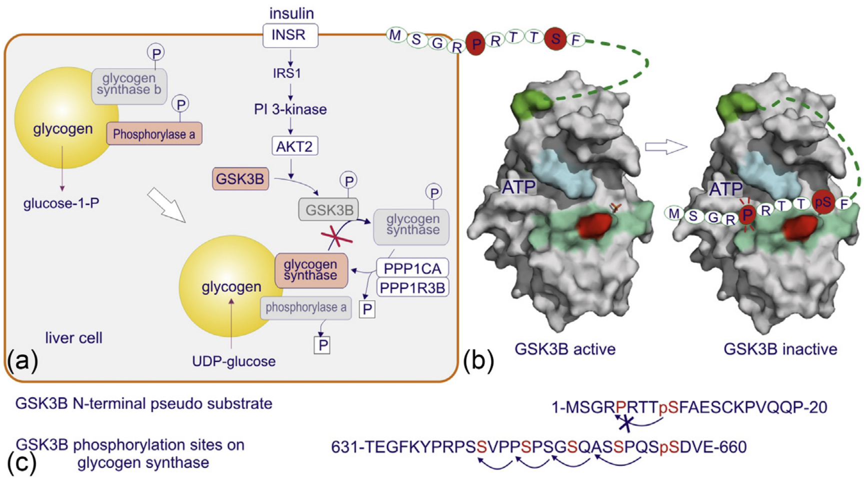


FIGURE 16-12 Insulin-mediated activation of glycogen synthase. (a) Binding of insulin to its receptors leads to the activation of AKT2. One of its substrates is glycogen synthase kinase-3β (GSK3B), which becomes inactivated, unable to phosphorylate and inactivate glycogen synthase. Also, the phosphatase PPP1CA ensures rapid dephosphorylation and activation of glycogen synthase allowing glycogen synthesis to recommence. In addition, phosphorylase-*a* is inactivated by dephosphorylation. Glycogenolysis is arrested. (b) Detail of the inhibition of GSK3B by AKT2. The N-terminal (pseudosubstrate) segment of GSK3B is most likely disordered, a wagging tail projecting out of the kinase. When phosphorylated (at serine-9) it takes on the characteristics of a primed substrate and binds at the catalytic cleft. However, lacking a second serine at a position four amino acids upstream the catalytic action is hindered and the peptide remains firmly attached (action of pseudosubstrate). Access of other substrates is prevented. (c) Comparison of the N-terminal sequence of GSK3B with the C-terminal sequence of glycogen synthase. The lack of a second (upstream) serine renders the phosphorylated N-terminal sequence a pseudo-substrate.

Signaling Disables the AXIN/APC Destruction Complex.") Phosphorylation of the most N-terminal serine by AKT then exposes a 10 residue pseudosubstrate sequence that neatly fits the catalytic cleft of GSK3B (see Figure 16-12(b)). Having a proline, not a serine four residues upstream of the phosphosite (pS), it blocks rather than activating kinase activity (Cross et al., 1995; Frame et al., 2001). Importantly, inactivation of GSK3B necessarily reduces phosphorylation at regulatory serine residues on glycogen synthase so that it becomes activated.

Alone, however, this is insufficient to halt the abrupt onset of glycogen synthesis. In order for this to occur, it is also necessary to activate the protein phosphatase that removes phosphate groups from glycogen synthase. Dephosphorylation occurs through PPP1CA which in the liver is bound to the glycogen-bound regulatory subunit PPP1R3B and muscle to PPP1R3A (or -F) (Figure 16-1). In muscle AKT2 phosphorylates and activates phosphodiesterase (PDE), the enzyme that removes cAMP (by conversion into

AMP). This inhibits the cAMP-dependent protein kinase A and prevents the action of glycogen phosphorylase (converts glycogen in multiple copies of glucose-1-P). The insulin-stimulated cell is now fully engaged in the synthesis of glycogen.

Diabetes mellitus, so named because the sufferers are permanently thirsty and produce large quantities of urine (they "syphon") that tastes of honey, affects 1%–2% of people in Western societies, being especially prevalent among Hispanic and native American peoples. Type 1 (or early onset) diabetes is due to autoimmune destruction of the insulin-producing β cells present in the islets of Langerhans in the pancreas and is a more severe form of the disease. It accounts for most instances of blindness in Western populations. Multiple genes are involved that determine susceptibility but not an inevitability to express the condition that might be precipitated by a viral infection.

The more common Type II (or late onset) form of diabetes appears to be a correlate of good living. It is associated with obesity though genetic factors undoubtedly play a part. It is characterized by resistance to insulin, in which an abundance of fatty acids plays an important part, and indeed, the early stages are characterized by very high levels of the hormone which, however, are insufficient to overcome the resistance.

Insulin induces translocation of the glucose transporter SLC2A4

Insulin is instrumental in the control of glucose uptake by the tissues, in particular muscle, fat, and those areas of the brain that regulate metabolic homeostasis (Benomar et al., 2006). It does this by the recruitment of the solute carrier SLC2A4 (GLUT4) to the plasma membrane from intracellular vesicular stores. In the absence of insulin, only ~5% of the total transporter pool is found on the cell surface. Exclusion of SLC2A4 from the cell surface depends on efficient sorting and sequestration into storage vesicles (a collection of endosomal vesicles, trans-Golgi network vesicles, and specialized storage vesicles) that do not readily recycle to the plasma membrane in the absence of stimulation but translocate there in response to insulin or exercise. Under these conditions glucose transport normally increases 10-fold. A reduced uptake due to a diminished membrane expression of SCL2A4 represents an early step in the development of insulin resistance and type II diabetes mellitus. About 90% of insulin-stimulated glucose uptake occurs in skeletal muscle, whereas adipose tissue and the liver accounts for the remaining 10% (Saltiel and Kahn, 2001). SLC2A4 is one of 14 members of the SLC2 family of facilitative-transmembrane sugar transporters, each of which has a distinct affinity and specificity for particular sugars, as well as unique tissue distribution, subcellular localization, and physiological function.

The insulin acts at two levels: (1) activating the docking machinery and (2) facilitating the transport of SCL2A4 vesicles to the prepared docking sites.

With respect to (1), the process starts by binding of the adaptor protein SH2B2 (formerly APS) to the tyrosine-phosphorylated insulin receptor. As the name indicates, it carries not only an SH2 but also a PH domain (Hu et al., 2006). This binds another adaptor SORBS1 which in turn is connected to CBL. The latter is phosphorylated by the receptor tyrosine kinase and this causes the activation of RAPGEF1. The guanine exchange protein loads the monomeric GTPases RHOQ with GTP. GTP-bound RHOQ interacts with EXOC7 and TRIP10, both of which are components of the exocyst complex (vesicle docking function). Once secretion vesicles have docked, they move to a membrane fusion site with SNARE proteins (Yokouchi et al., 1999; Chang et al., 2006).

With respect to (2), the sequence of events starts with AKT2-mediated phosphorylation and inactivation of the Rap-GTPse TBC1D4 (formerly AS160) thereby promoting the GTP-bound state of RAP GTPases (RAB2A, 8A, 10, 14, are concerned). RAB10 directly facilitates translocation and plasma-membrane docking of specialized SCF2A4 vesicles, whereas RAB14 mediates delivery of vesicles via endosomal compartments (membrane structures that co-express transferrin receptors). Moreover, RAP10 plays a role in connecting the specialized SCF2A4 vesicles with the cortical actin network by recruiting the unconventional myosin-Vα (MYO5A) (Chen et al., 2012; Foley et al., 2011) (return to Figure 16-1 for a schematic representation of events). For more information about the role of SNAREs in membrane fusion, return to Chapter 6, “Intracellular Calcium,” Section “Ca^{2+} in action,” Figure 6-20.

THE ROLE OF PI3-KINASE IN ACTIVATION OF PROTEIN SYNTHESIS

Insulin is a growth factor. Again, operating through the PI3-kinase pathway, it increases protein synthesis by a mechanism that involves intermediates including the GTPase RHEB and that acts upon the initiator factor-4E (EIF4E) and the ribosomal S6-kinase-1 (RPS6KB1).

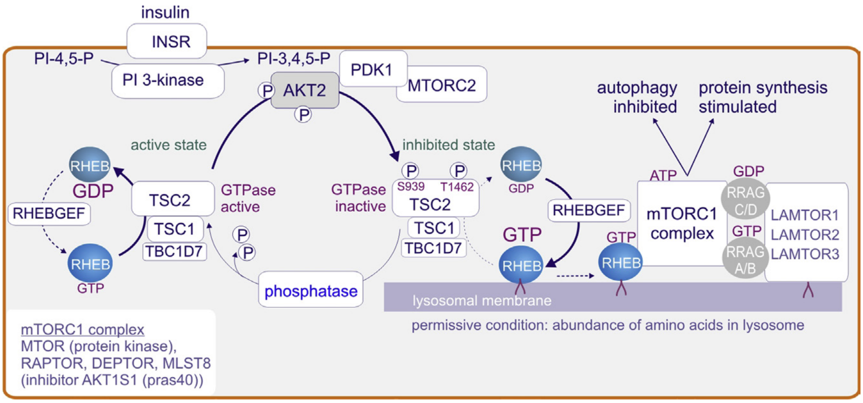


FIGURE 16-13 Regulation of mTORC1 by AKT-mediated phosphorylation of TSC2. The complex TSC1/TSC2/TBC1D7 acts as a GAP, maintaining Rheb in its (GDP) inactive state and holding protein synthesis in check. Insulin augments the rate of protein synthesis through activation of Rheb. This occurs through phosphorylation and inactivation of TSC2 by AKT2. Rheb then accumulates in its GTP-bound state (probably involving an as yet unidentified GEF) and interacts with the kinase mTOR, complexed to Raptor (and GβL which is not shown). This unveils its kinase activity and causes the complex to associate with eIF3.

We return to the role of RHEB and its immediate effector in the following paragraphs.

EIF4E is the limiting initiation factor in most cells and it plays a principal role in determining global translation rates. It is regulated by phosphorylation, for instance through the ERK pathway (return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases" Section "Regulation of Protein Synthesis Through the ERK–MNK1 Pathway") but more importantly by binding to the translational repressor EIF4EBP (EIF4-binding protein). EIF4E binds to the 7-methylguanine (m7G) cap at the 5′-end of mRNA and enables the recruitment of other initiation factors. These, together with the 40S ribosomal subunit, form the initiation complex which promotes the search for the start codon (AUG) on the mRNA (Figure 16-14). Progression is facilitated by the helicase complex EIF4A/EIF4B which breaks the intramolecular base pairing that occasionally occurs in the mRNA. Recognition of the AUG codon requires $tRNA^{Met}$ that contains the anticodon 3′-UAC-5′ and comes into the ribosomal pre-initiation complex bound to EIF2S3 in its GTP-bound state (formerly eIF-2γ). As the AUG codon is recognized, GTP is hydrolyzed and the ribosomal 60S particle, carrying the peptidyl transferase activity, joins the initiation complex, thereby initiating the elongation phase of protein synthesis. Binding of EIF4EBP1 (abbreviated to 4EBP in images) to eIF4E prevents all this.

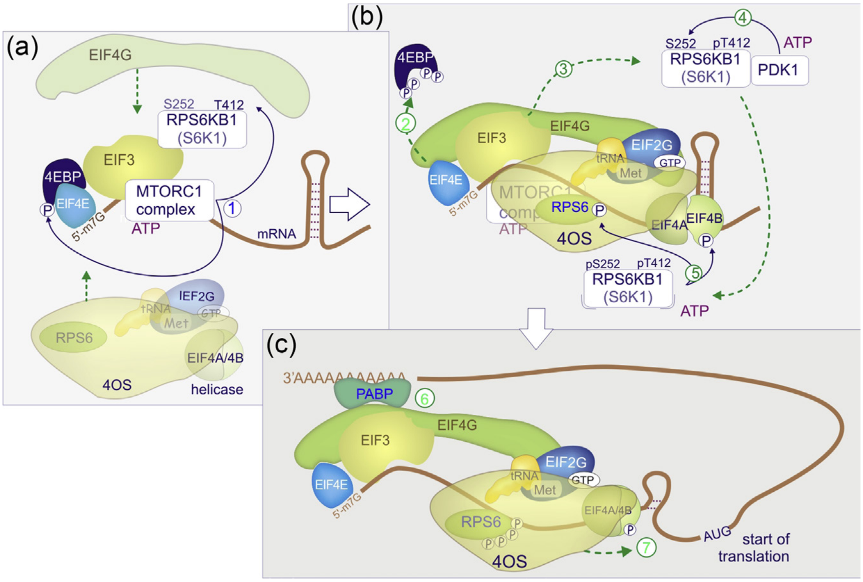


FIGURE 16-14 A series of ordered phosphorylations facilitates assembly of the translation initiation complex on mRNA. (a) Activation and association of mTOR, as part of MTORC1 complex, with the initiation complex causes phosphorylation of EIF4EBP1 (4EBP) (1) and of RPS6KB1 (2). Phosphorylated EIF4EBP1 detaches from EIF4E and phosphorylated RPS6KB1 detaches from EIF3. NB: EIF-3 and the 40S ribosomal particle are multicomponent complexes represented as single entities. (b) Dissociation of EIF4EBP1 (2) and RPS6KB1 (3) permits the association of the large initiation factor EIF4G that interacts with EIF4E, EIF3, and EIF4A/B. RPS6KB1, phosphorylated in its regulatory domain (including the hydrophobic motif), encounters PDK1 (4) causing phosphorylation of its activation segment. RPS6KB1 now phosphorylates the ribosomal proteins S6 (RPS6) and EIF4B, the regulatory subunit of the DNA helicase EIF4A (5). (c) The conditions now favor binding of poly-A-binding protein (PABP) which is attached to the 3′-poly A tail of the mRNA (6). The initiation complex moves toward the start AUG codon (7) where it is joined with the 60S ribosomal particle.

In response to insulin and to numerous growth factors, the RPS6KB1 initially associated with EIF3 (a large complex of small initiation factors), acts to phosphorylate the ribosomal S6 particle, RBS6, a component of the 40S ribosomal subunit (Novak-Hofer and Thomas, 1985; Smith et al., 1980). Cells lacking RBS6 are smaller in size but how it actually regulates cell size remains an enigma. Cellular localization of RBS6 is not restricted to the 40S ribosomal particle, so it may control cell size independent of protein synthesis at the ribosomal level (Ruvinsky and Meyuhas, 2006). RPS6KB1 also phosphorylates the regulatory domain of EIF4A. As we will see below, the critical task of eliminating EIF4EBP1 is taken care of by the mTORC1 complex.

The S6 protein kinases form a subfamily of serine/threonine protein kinases which comprises the following members: RPSKA1–6, and RPS6KB1, and B2. The S6 kinase discussed here represents RPSKB1. It phosphorylates the ribosomal S6 protein (RPS6) at six serine residues. Two of these are also phosphorylated by RPS6KA1 (formerly RSK), a protein-kinase downstream of the RAS/MAPK pathway.

Tuberous sclerosis (TSC). It was first described by Désiré-Magloire Bourneville in 1880 (and called Bourneville's disease in France) (Bourneville, 1880; Bourneville and Brissaud, 2008). TSC is a rare (30–100 per million, worldwide), multisystem genetic disease characterized by the growth of benign tumors in the brain and in other vital organs including the kidneys, heart, eyes, lungs, and skin. It is caused by mutations on either of two TSC genes, 1 or 2, which encode for the proteins hamartin and tuberin, respectively. These act as tumor suppressors, regulating cell proliferation and differentiation. PTEN, which acts in the same signal transduction pathway, is also a tumor suppressor but loss of its function is linked to Cowden syndrome in which patients have a predisposition to malignancies such as prostate cancer, glioblastoma, endometrial tumors, and small-cell lung carcinoma (Cantley and Neel, 1999).

RHEB AND TSC

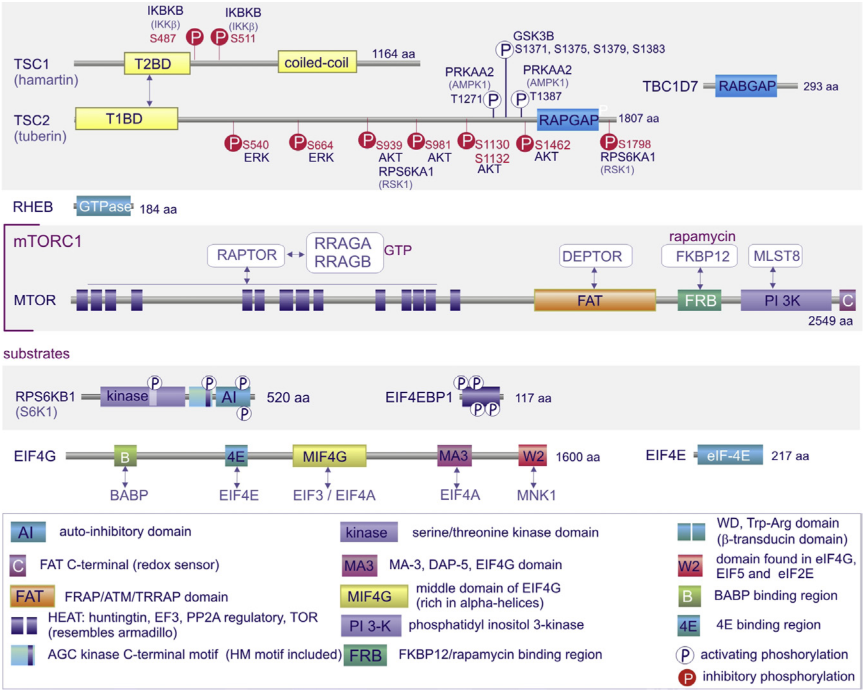
TSC1 and TSC2 are downstream components of the PI3-kinase pathway, discovered as loss-of-function mutants in patients suffering from tuberous sclerosis (Cheadle et al., 2000; van Slegtenhorst et al., 1997; The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). TSC1/TSC2 binds a third partner TBC1D7 (Dibble et al., 2012). The complex constitutes a GTPase-activating quality (intrinsic property of both TSC2 and TBCD1D7) that holds the monomeric GTPase RHEB in its GDP-bound inactive state (Inoki et al., 2003). Phosphorylation of TSC2 (in particular on residues S939 and T1462) by AKT2 inhibits this activity so allowing accumulation of GTP-bound RHEB that now it is in the position to bind and activate the mTORC1 complex (Figure 16-13) (Inoki et al., 2002; Dan et al., 2002).

The kinase MTOR takes part in the MTOR complex-1 (mTORC1) together with four regulatory subunits RPTOR (Raptor), DEPTOR, and MLST8 (formerly GβL), and AKT1S1 (formerly Pras40). It binds to EIF3 and coordinates the assembly of the initiation complex through a series of ordered phosphorylation events. First, mTORC1 phosphorylates EIF-4EBP1, at multiple sites, causing its detachment from EIF4 (Gingras et al., 2001) and allowing the association of EIF4G with the initiation complex. Importantly, binding of EIF4G causes the interaction of the 5′-mRNA initiation complex with the 3′ mRNA poly-A-binding proteins (BAPB). This critically drives translation, ensures protection against exonucleases, and, importantly, ensures that only intact mRNA is translated. Next mTORC1 contributes to the activating process of RPS6KB1 (Burnett et al., 1998).

It is not fully established yet in which order, but RPS6KB1 requires three phosphorylation steps, one in the hydrophobic motif, one in the turn motif (probably due to auto-phosphorylation), and one in the activation segment. In Figure 16-14 we illustrate the sequence where mTORC1 first phosphorylates the hydrophobic motif (T412), followed by a PKD1-mediated phosphorylation of the activation segment (T252) (Wang et al., 2013; Keshwani et al., 2011). In the process, PDK1 dissociates from EIF3 and, once active, it phosphorylates the S6 ribosomal protein and also EIF4B (Holz et al., 2005). As EIF2S3 in its GTP-bound state GTP (eIF-2γ) and tRNA$^{\text{Met}}$ combines with the initiation complex, the small ribosomal subunit starts its progression toward the 3′end. It halts when it attains the AUG codon, binding to the anticodon of tRNA$^{\text{Met}}$. Protein translation commences. The domain architecture of the components of the TSC/mTORC1 pathway is depicted in Figure 16-15.

> The loss-of-function mutations in TSC1 and TSC2 (also called harmatin and tuburin, respectively) give rise to hamartomas, benign tumors having the form of nodules or tubers, in brain, heart, skin, and kidney. TSC1 and 2 qualify as tumor suppressors. PTEN, which acts in the same signal transduction pathway, is also a tumor suppressor but loss of its function is linked to Cowden syndrome in which patients have a predisposition to malignancies such as prostate cancer, glioblastoma, endometrial tumors, and small-cell lung carcinoma.

Although insulin-mediated activation of the MAPK pathway is without significant effect on glucose transport or glycogen synthesis, it does have a significant effect on protein synthesis (Yamamoto-Honda et al., 1995; Dorrestijn et al., 1996). This occurs through ERK-mediated (MAPK1 and -3) phosphorylation of TSC2 at sites distinct from those targeted by AKT2 and it also causes inhibition of the GAP activity (Ma et al., 2005). The MAPK pathway also causes phosphorylation through one of the downstream protein kinases, RPS6KA1 (formerly RSK1) (Roux et al., 2004). For details of some of the phosphorylation sites, see Figure 16-15.



AI	auto-inhibitory domain	kinase	serine/threonine kinase domain	▮▮	WD, Trp-Arg domain (β-transducin domain)
C	FAT C-terminal (redox sensor)	MA3	MA-3, DAP-5, EIF4G domain	W2	domain found in eIF4G, EIF5 and eIF2E
FAT	FRAP/ATM/TRRAP domain	MIF4G	middle domain of EIF4G (rich in alpha-helices)	B	BABP binding region
▮▮	HEAT: huntingtin, EF3, PP2A regulatory, TOR (resembles armadillo)	PI 3-K	phosphatidyl inositol 3-kinase	4E	4E binding region
▮	AGC kinase C-terminal motif (HM motif included)	FRB	FKBP12/rapamycin binding region	Ⓟ	activating phoshorylation
				Ⓟ	inhibitory phosphorylation

FIGURE 16-15 Domain architecture of the components of the mTOR signaling pathway. Many of the components of the mTOR pathway are large proteins, some having still poorly defined domains. TSC2 carries a GAP domain responsible for activation of hydrolysis of GTP by RHEB. The numerous phosphorylation sites have either stimulatory effects (sites phosphorylated by GSK3B and PRKAA2), or inhibitory effects (in red). mTOR bears the signature sequence of a PI3-kinase but operates as a protein kinase. It forms a complex, mTORC1, with RAPTOR, DEPTOR, and MLST8. In the presence of rapamycin, it also binds FKBP12 which inhibits kinase activity. Note that the GTPases RRAGA/B recruit the mTORC1 complex to the lysosomal membrane through binding to RAPTOR. RPS6KB1 has an auto-inhibitory and an AGC-kinase C-terminal domain with hydrophobic motif, both of which have to be phosphorylated before phosphorylation and activation of the activation segment. IEF4EBP1 has to be phosphorylated at four residues before it effectively detaches from EIF4E. We show EIF4G because it acts as a big scaffold to which a number of components of the initiation complex are attached. Note the interaction with MNK1, a member of MAP-kinase family. *Information about phosphorylation sites on TSC2 are obtained from Huang and Manning (2008).*

INTEGRATION OF GROWTH FACTOR AND NUTRIENT SIGNALING

Amino acids have a permissive role in mTORC1 activation (facilitating RHEB-mediated activation)

The activation process described above is abrogated when cells are deprived of amino acids for the simple reason that RHEB and mTORC1 do not get the chance to meet. Amino acids, particularly leucine and arginine, must be present for any upstream signal, including growth factors, to activate mTORC1 (Blommaart et al., 1995). Besides RHEB, activation of TORC1 also requires the presence of the monomeric GTPases of the Ras-related RRAG family (Kim et al., 2008; Sancak et al., 2008). The RRAG family comprises four members, all expressed in mammalian cells where they act in obligate pairs, RRAGA with RRAGB and RRAGC with RRAGD. The two dimers appear to have opposite nucleotide loading states, so that when RRAGA/B is bound to GTP, RRAGC/D is bound to GDP and vice versa. Through an unknown mechanism, amino acids promote the loading of RRAGA/B with GTP, which enables the heterodimer to interact with the RPTOR component of mTORC1. This interaction results in the translocation of mTORC1 from a poorly characterized cytoplasmic location to the lysosomal surface, where the Rag GTPases dock on a multi-subunit complex composed of LAMTOR1-3 and named "Ragulator" (Sancak et al., 2010; Demetriades et al., 2014). At the membrane, mTORC1 effectively meets the membrane-bound RHEB (it possesses a farnesyl group). Both buttons need to be activated (RHEB and RRAG) in order to firmly attach mTORC1 to the membrane compartment and cause maximal activation. Active mTORC1 inhibits autophagy and promotes protein synthesis (Figure 16-13). When the lysosomes lack amino acids, as we assume may occur during starvation, RRAGA/B return to the GDP-bound, and RRAGC/D to the GTP-bound state, and they fail to hold mTORC1 at the membrane thus preventing the stimulatory action of RHEB. Below we show that high levels of AMP also negatively regulate mTORC1 but in a different fashion.

We note that LAMTOR1–3 also qualify as scaffold proteins for the MAP-kinase signaling cassette positioned at the late endosomes. They play a role in the continuation of growth factor signaling after endocytosis of the receptor complex (Wunderlich et al., 2001; Teis et al., 2002). Return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Section "Mammalian Scaffold Protein that Regulates MAP-Kinase Signaling."

High levels of AMP, a hallmark of ATP depletion, inhibit mTORC1

The presence of 5′-AMP, which indicates a low concentration of ATP, must intervene with the growth factor action of insulin in order to prevent unfinished production of proteins due to ATP insufficiency (ATP feeds both ATP- and GTP-dependent processes) (Shamji et al., 2003). Regulation by 5′-AMP occurs at the level of the 5′-AMP-sensitive protein kinase which occurs as a complex of three proteins: the catalytic subunit PRKAA2, a 5′-AMP-binding regulatory γ-subunit PRKAG1, and a small regulatory β-subunit PRK1B1 (Figure 16-16). 5′-AMP is

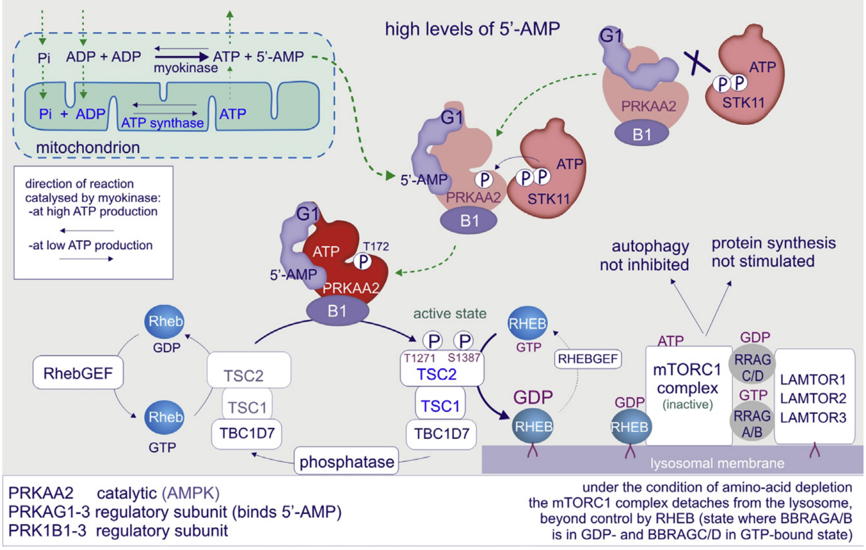


FIGURE 16-16 Signaling through the AMP-sensitive protein kinase. Hints of ATP depletion are manifested by the leakage of 5′-AMP from mitochondria which binds the regulatory γ-subunit of the 5′-AMP-sensitive protein kinase (PRKAG1). Subsequent phosphorylation of the activation segment of the catalytic subunit PRKAA2 by STK11 leads to catalytic competence. The 5′-AMP-sensitive kinase phosphorylates TSC2 (residues T1271 and S1387) thereby preventing the inhibitory control by AKT2. The active TSC1/2 complex maintains RHEB in its GDP-bound state thus preventing activation of mTORC1. Protein synthesis is suppressed and autophagy is no longer prevented. In the case where the cells also lack certain amino acids (starvation), the mTORC1 complex is no longer bound to the membrane surface and this prevents the case where even residual levels of GTP-bound RHEB may drive protein synthesis.

not a direct activator of the 5′-AMP-sensitive kinase in the way that cyclic AMP activates protein kinase A. Instead, similar to the activation of AKT by PIP_3, it acts as a recruiting agent. By binding to the γ-regulatory subunit (PRKAG1), 5′-AMP induces a conformational change that allows the interaction of the enzyme with STK11 (formerly LKB1), a constitutively activated kinase. This results in phosphorylation of the activation segment and activation of PRKAA2. The importance of this pathway in the regulation of protein synthesis is illustrated by the finding that lack of STK11 activity predisposes to cancer (Peutz–Jeghers syndrome). STK11 thus qualifies as a tumor suppressor. It prevents excessive stimulation of the mTORC1 pathway and in this way it maintains strict control over protein synthesis (Alessi et al., 2006). Further evidence for a tumor suppressor role of STK11 comes from the finding that its activation, through the induction of its pseudokinase partner STRAD causes repolarization of transformed epithelial cells with the reformation of tight junctions. STK11 thus appears to classify as both a polarity and a tumor suppressor gene (Baas et al., 2004). Activation of 5′-AMP-sensitive kinase leads to phosphorylation of the TSC2 on residues distinct from those phosphorylated by all the aforementioned protein kinases, namely at T1271 and S1387 and these prevent the "inhibitory phosphorylations" caused by AKT2 (return to Figure 16-15). As a consequence, the GAP activity remains intact and RHEB exists in a predominant GDP-bound state, incapable of activing mTORC1.

The TSC1/TSC2/TBC1D7 complex is one of a number of typical examples that illustrates how certain components of signal transduction pathways are able to integrate a fast array of upstream signals. Together, they act as a true molecular switchboard in the control of protein synthesis. We have discussed the action of AKT and PRKAA2 (AMPK) but other kinases have their say as well. Phosphorylation sites have been detected for CDK1 (control of cell cycle), IKBKB (inflammation), MAPK members (MAPK1, -3, MAPKAPK2, RP66KA1, all downstream of growth factors), and GSK3B (acting downstream of Wnt signaling). In human TSC1, phosphorylation has been detected for 23 serines, 11 threonines, and 8 tyrosines whereas K751 has been found acetylated and K752 ubiquitinylated. Human TSC2 counts 27 serine, 7 threonine, and 7 tyrosine phosphorylation sites to which can be added, 3 acetylation and 5 ubiquitinylation sites. Posttranslational modifications have also been detected for TBC1D7, a small protein with only a modest score of 3 ubiquitinylation, 1 phosphoserine, and 2 phosphotyrosine sites. Source: Information from www.phosphosite.org and from Huang and Manning (2008).

PI3-KINASE, REGULATOR OF CELL SIZE, PROLIFERATION, AND TRANSFORMATION

PIK3CA and PIK3CB (class I, p110α, and -β) are implicated in cancer. For instance, activating mutations or mutations that cause overexpression of PIK3CA are associated with colorectal (MIM 114500), breast (MIM 114480), ovarian (MIM 167000), hepatocellular (MIM 114550), and skin cancer (MIM 182000). It is also associated with Cowden syndrome (MIM 615108) (source UniProt and Ref. Samuels and Velculescu, 2004). Indeed, after KRAS, it is the most mutated gene implicated in cancer. On the other hand, activating mutations in AKT are not associated with human cancers though overexpression has been detected (particularly AKT), again predominantly in colorectal cancers.

Web Resource

Find out more about the cancers on the OMIM Website. URL: www.ncbi.nlm.nih.gove/omim.

The first indication that PI3-kinase might act as a regulator of cell size and proliferation came from studies with *Drosophila*. Overexpression of Dp110 (catalytic subunit of PI3-kinase) results in enlarged wings and eyes. Conversely, kinase defective mutants have very small organs (Leevers et al., 1996) (Figure 16-17). The PI3-phosphatase, PTEN, correspondingly reduces both cell size and number (Goberdhan et al., 1999). AKT stimulates those pathways that specifically regulate cell and compartment size independently of cell number (Verdu et al., 1999). Why are PTEN inactivation mutants so often linked to malignancy, whereas TSC1/2 mutants only rarely so, after all they both operate in the same pathway? PTEN has many roles. Not only does it regulate cell size, but it is also a determinant of cell survival and it regulates the cell cycle through expression of cyclin D (acting in the G1 phase). Indeed, this may explain why its loss of function gives rise to a "higher level" of cell transformation. A second explanation has emerged from a recent study in which mice lacking TSC2 manifest deficient signaling of the AKT pathway, and this may limit the transforming capacity. However, loss of a single copy of the PTEN gene, so-called haplodeficiency, restores the activation level of AKT and with it, its transforming consequences (Manning et al., 2005). Overexpression of EIF4E can induce full tumorigenic transformation (Lazaris-Karatzas et al., 1990). It also leads to enlarged cells and increased proliferation rates and it can rescue protein synthesis even when mTORC1 is inhibited by rapamycin (Fingar et al., 2002). Not surprisingly, the PI3-kinase/mTORC1 pathway is regarded as a promising target for therapeutic intervention and many derivatives of rapamycin are now being tested both in animal models and in clinical trials. A list of oncogenes and tumor suppressors linked to the PI3-kinase/mTOR pathway is presented in Table 16-1.

That the understanding of how tumors arise is impressive but still limited as is given by the observation that mice lacking the tyrosine phosphatase PTPN1 (formerly PTP1B) exhibit no predisposition to cancer. This, despite the fact that this phosphatase has a central place in the deactivation of the insulin receptor and that when absent, the AKT signaling pathway is strongly enhanced. These mice are, however, highly responsive to insulin and show little propensity to obesity (Elchebly et al., 1999). We return to PTPN1 in Chapter 18, "Protein Phosphatases."

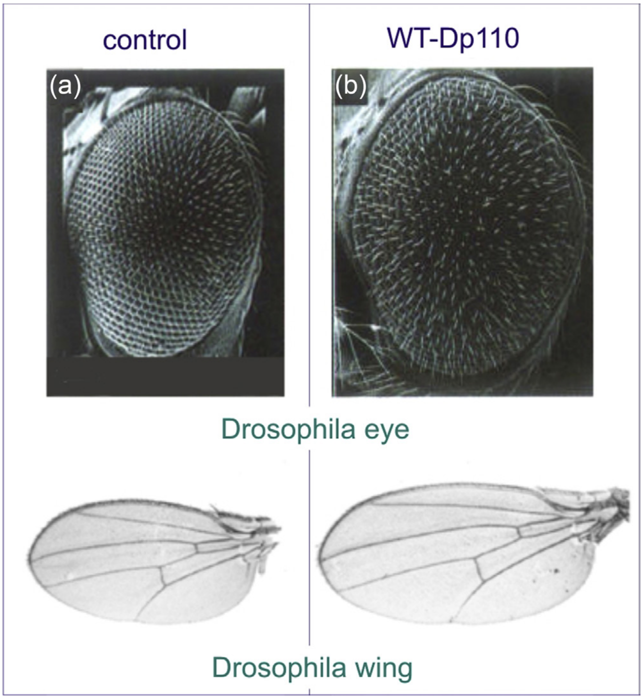


FIGURE 16-17 Overexpression of *Drosophila* type I PI3-kinase (Dp110) yields flies with giant eyes (and wings). *From Ref.* *Leevers et al. (1996)*.

mTORC1 represents the rapamycin-sensitive complex. Rapamycin is a triene macrolide antibiotic obtained in *Streptomyces hygroscopius*, first dug up out of the earth of Rapa Nui (Easter Island). It binds to a cellular receptor FKBP12 and, together, they form a complex with RPTOR (regulatory-associated protein of mTOR) which is bound to mTOR in the mTORC1 complex. This prevents interaction with substrates. Such inhibitory complex formation does not occur when MTOR is associated with RICTOR (rapamycin-insensitive companion of MTOR) (mTORC2).

Suggested reviews about mTOR:

- Huan, J., Manning, B., 2008. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. Biochem. J. 412, 179–190.
- Ma, X.M., Blenis, J., 2009. Molecular mechanisms of mTOR-mediated translational control. Nat. Rev. Mol. Cell. Biol. 10, 307–318.

continued

- Russell, R.C., Gang, C., Guan, K.-L., 2011. An emerging role for TOR signaling in mammalian tissues and stem cell physiology. Development 138, 3343–3356.
- Laplante, M., Sabatine, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274–293.
- Dibble, C.C., Manning, B.D., 2013. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. Nat. Cell. Biol. 15, 555–564.

The transcriptional control of metabolism, including the key role of SREBP1 in fatty acid synthesis is reviewed in:

- Desvergne, B., Michalik, L., Wahli, W., 2006. Transcriptional regulation of metabolism. Physiol Rev 86, 465–514.

TABLES 16-1 Proto-oncogenes and tumor suppressors associated with the mTOR/Raptor (mTORC1)-signaling pathway

Proto-oncogenes associated with the mTORC1 signaling pathway	
PI 3 kinase	Elevated activity in multiple cancers
AKT	Amplified gene expression is some cancers
RHEB	Elevated expression, possible target of farnesyl transferase inhibitors
RAS	Activating mutations
EIF4E	Over expressed in many cancers
RS S6 kinase	Elevated expression in breast cancer
Tumor-suppressor functionally linked to mTORC1 signaling	
PTEN	Loss-of-function in numerous cancers and in Cowden syndrome
TSC1/TSC2	Loss-of-function causes hamartomas in several organs
NF1	Mutations in neurofibromatosis type-1
STK11 (LKB1)	Mutations in Peutz–Jeghers syndrome
EIF4BP1	Overexpression blocks MYC-induced oncogenic transformation

OTHER PROCESSES MEDIATED BY THE 3-PHOSPHORYLATED INOSITOL PHOSPHOLIPIDS

A number of seemingly disparate functions are modified in cells and organisms in which the synthesis of the 3-phosphorylated lipids is chronically altered. These include:

- A constitutively activated retrovirus-encoded PI3-kinase induces transformation of fibroblasts—in chickens, formation of hemangiomas.

- Inhibition of PI3-kinase prevents T-cell activation by preventing the translocation of NFAT.
- In vascular endothelial cells subjected to shear stress, nitric oxide synthase is activated by AKT. The released NO relaxes the vascular smooth muscle (Dimmeler et al., 1999).
- RAC1 and CDC42 cooperate with PI-4-P-5-kinase and PI3-kinase in the assembly of the submembranous actin filament system leading to the formation of the membrane protrusions necessary for neutrophil migration (Kwiatkowska and Sobotka, 1999). Conversely, membrane retraction occurs through localized activation of PTEN at the trailing edge of migrating neutrophils. This pathway involves RhoA-mediated activation of Rock, which in turn phosphorylates and activates PTEN (Xu et al., 2003; Li et al., 2003).
- In *C. elegans*, akt-1 phosphorylates and activates a transcription factor of the forkhead/winged-helix family (Kops et al., 1999). This has a function in resisting apoptosis and may regulate lifespan. Mutants having reduced activity of the insulin/IGF1-receptor homologue daf-2 and therefore unable to activate PI3-kinase live twice as long as normal counterparts.
- In epithelial cells, activation of the apoptosis pathway is suppressed as a consequence of PI3-kinase activation (return to Chapter 11, “Signal Transduction to and from Adhesion Molecules,” Section “Integrins and cell survival”).

So, who did discover insulin?

Probably “nobody discovered insulin.” It existed first as an idea (Minkowski), then as a proven hypothesis (Gley and others), and finally as a practical way of alleviating diabetes (Paulescu and Banting). ***Adapted from Henderson, 1971.***

References

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., Cantley, L.C., 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 57, 167–175.

Alessi, D.R., Sakamoto, K., Bayascas, J.R., 2006. LKB1-Dependent signaling pathways. Ann. Rev. Biochem. 75, 137–163.

Bellacosa, A., Testa, J.R., Staal, S.P., Tsichlis, P.N., 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science 254, 274–277.

Benomar, Y., Naour, N., Aubourg, A., Bailleux, V., Gertler, A., Djiane, J., Guerre-Millo, M., Taouis, M., 2006. Insulin and leptin induce Glut4 plasma membrane translocation and glucose uptake in a human neuronal cell line by a phosphatidylinositol 3-kinase-dependent mechanism. Endocrinology 147, 2550–2556.

Bourneville, D.M., 1880. Sclérose tubéreuse des circonvolutions cérébrales. Arch. Neurol. Paris 1, 81–89.

Bourneville, D.M., Brissaud, E., 2008. Encéphalite ou sclérose tubéreuse des circonvolutions cérébrales. Arch. Neurol. Paris 1, 390–410.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., Sabatini, D.M., 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc. Natl. Acad. Sci. U.S.A. 95, 1432–1437.

Blommaart, E.F., Luiken, J.J., Blommaart, P.J., van Woerkom, G.M., Meijer, A.J., 1995. Phosphorylation of ribosomal protein S6 is inhibitor for autophagy in isolated rat hepatocytes. J. Biol. Chem. 270, 2320–2326.

Baas, A.F., Kuipers, J., van der Wel, N.N., Batlle, E., Koerten, H.K., Peters, P.J., Clevers, H.C., 2004. Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. Cell 116, 457–466.

Chong, L.D., Traynor-Kaplan, A., Bokoch, G.M., Schwartz, M.A., 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell 79, 507–513.

Closse, A., Haefliger, W., Hauser, D., 1981. 2,3-Dihydrobenzofuran-2-ones: a new class of highly potent anti-inflammatory agents. J. Med. Chem. 24, 1465–1471.

Calleja, V., Alcor, D., Laguerre, M., Park, J., Vojnovic, B., Hemmings, B.A., Downward, J., Parker, P.J., Larijani, B., 2007. Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. PLoS Biol. 5, e95.

Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, et al., 2007. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 448, 439.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

Chang, L., Chiang, S., Saltiel, A.R., 2006. Insulin signaling and the regulation of glucose transport. Mol. Med. 10, 65–71.

Chen, Y., Wang, Y., Zhang, J., Deng, Y., Jiang, L., Song, E., Wu, X.S., Hammer, J.A., Xu, T., Lippincott-Schwartz, J., 2012. Rab10 and myosin-Vα mediate insulin-stimulated GLUT4 storage vesicle translocation in adipocytes. J. Cell Biol. 198, 545–560.

Cantley, L.C., Neel, B.G., 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 4240–4245.

Cheadle, J.P., Reeve, M.P., Sampson, J.R., Kwiatkowski, D.J., 2000. Molecular genetic advances in tuberous sclerosis. Hum. Genet. 107, 97–114.

Diekmann, D., Brill, S., Garrett, M.D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., Hall, A., 1991. Bcr encodes a GTPase-activating protein for p21rac. Nature 351, 400–402.

Dann, S.G., Thomas, G., 2006. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett. 580, 2821–2829.

Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., Manning, B.D., 2012. TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. Mol. Cell 47, 535–546.

Dan, H.C., Sun, M., Yang, L., Feldman, R.I., Sui, X.M., Ou, C.C., Nellist, M., Yeung, R.S., Halley, D.J., Nicosia, S.V., Pledger, W.J., Cheng, J.Q., 2002. Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J. Biol. Chem. 277, 35364–35370.

Dorrestijn, J., Ouwens, D.M., Van den Berghe, N., Bos, J.L., Maassen, J.A., 1996. Expression of a dominant-negative Ras mutant does not affect stimulation of glucose uptake and glycogen synthesis by insulin. Diabetologia 39, 558–563.

Demetriades, C., Doumpas, N., Teleman, A.A., 2014. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. Cell 156, 786–799.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., Zeiher, A.M., 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., et al., 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283, 1544–1548.

Li, Z., Hannigan, M., Mo, Z., Liu, B., Lu, W., Wu, Y., Smrcka, A.V., Wu, G., Li, L., Liu, M., Huang, C.K., Wu, D., 2003. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell 114, 215–227.

Freychet, P., 1990. Pancreatic hormones. In: Baulieu, E.-E., Kelly, P.A. (Eds.), Hormones: From Molecules to Disease. Chapman and Hall, London, pp. 490–532.

Foster, D.W., McGarry, J.D., 2006. Glucose, lipid and protein metabolism. In: Griffin, J.E., Ojeda, S.R. (Eds.), Textbook of Endocrine Physiology. Oxford University Press, New York, pp. 393–419.

Foster, F.M., Traer, C.J., Abraham, S.M., Fry, M.J., 2003. The phosphoinositide (PI) 3-kinase family. J. Cell Sci. 116, 3037–3040.

Frodin, M., Antal, T.L., Dummler, B.A., Jensen, C.J., Deak, M., Gammeltoft, S., Biondi, R.M., 2002. Phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. EMBO J. 21, 5396–5407.

Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., Tsichlis, P.N., 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81, 727–736.

Franke, T.F., Kaplan, D.R., Cantley, L.C., Toker, A., 1997. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 275, 665–668.

Frame, S., Cohen, P., Biondi, R.M., 2001. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol. Cell 7, 1321–1327.

Foley, K., Boguslavsky, S., Klip, A., 2011. Endocytosis, recycling, and regulated exocytosis of glucose transporter 4. Biochemistry 50, 3048–3061.

Fingar, D.C., Salama, S., Tsou, C., Harlow, E., Blenis, J., 2002. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. 16, 1472–1487.

Gley, E., 1922. Action des extraits de pancréas sclérosé sur des chiens diabétiqués (par extirpation du pancréas). C. R. Soc. Biol. Paris 87, 1322–1325.

Gary, J.D., Wurmser, L.S., Emr, S.D., 1998. Fap1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. J. Cell Biol. 143, 65–79.

Gunther, R., Abbas, H.K., Mirocha, C.J., 1989. Acute pathological effects on rats of orally administered wortmannin-containing preparations and purified wortmannin from Fusarium oxysporum. Food Chem. Toxicol. 27, 173–179.

Guinebault, C., Payrastre, B., Racaud, S.C., Mazarguil, H., Breton, M., Mauco, G., Plantavid, M., Chap, H., 1995. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 a with actin filaments and focal adhesion kinase. J. Cell Biol. 129, 831–842.

Gingras, A.C., Raught, B., Sonenberg, N., 2001. Regulation of translation initiation by FRAP/mTOR. Genes Dev. 15, 807–826.

Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M., Wilson, C., 1999. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev. 13, 3244–3258.

Henderson, J.R., 1971. Who really discovered insulin? Guy's Hosp. Gaz. 85, 314–318.

Honda, A., Nogami, M., Yokozeki, T., et al., 1999. Phosphatidylinositol 4-phosphate 5-kinase is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell 99, 521–532.

Herman, P.K., Stack, J.H., DeModena, J.A., Emr, S.D., 1991. A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell 64, 425–437.

Van Haesebroeck, B., Jones, G.E., Allen, W.E., Zicha, D., Hooshmand, R.R., Sawyer, C., Wells, C., Waterfield, M.D., Ridley, A.J., 1999. Distinct PI(3)Ks mediate mitogenic signaling and cell migration in macrophages. Nat. Cell Biol. 1, 69–71.

Hornbeck, P.V., Chabra, I., Kornhauser, J.M., Skrzypek, E., Zhang, B., 2004. PhosphoSite: a bioinformatics resource dedicated to physiological protein phosphorylation. Proteomics 4, 1551–1561.

Hu, J., Liu, J., Ghirlando, R., Saltiel, A.R., Hubbard, S.R., 2006. Structural basis for recruitment of the adaptor protein APS to the activated insulin receptor. Mol. Cell 12, 1379–1389.

Holz, M.K., Ballif, B.A., Gygi, S.P., Blenis, J., 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell 123, 569–580.

Huang, J., Manning, B.D., 2008. The TSC1-RSC2 complex: a molecular switchboard controlling cell growth. Biochem. J. 412, 179–190.

Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., Cossart, P., 1996. A role for phosphoinositide 3-kinase in bacterial invasion. Science 274, 780–782.

Inoki, K., Li, Y., Xu, T., Guan, K.L., 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 17, 1829–1834.

Inoki, K., Li, Y., Wu, J., Guan, K.L., 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signaling. Nat. Cell Biol. 4, 648–657.

Jones, P.F., Jakubowicz, T., Pitossi, F.J., Maurer, F., Hemmings, B.A., 1991. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc. Natl. Acad. Sci. U.S.A. 88, 4171–4175.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., Czech, M.P., 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc. Natl. Acad. Sci. U.S.A. 100, 7569–7574.

Kasuga, M., Karlsson, F.A., Kahn, C.R., 1982. Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science 215, 185–187.

Keshwani, M.M., von Daake, S., Newton, A.C., Harris, T.K., Taylor, S.S., 2011. Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). J. Biol. Chem. 286, 23552–23558.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., Guan, K.L., 2008. Regulation of TORC1 by Rag GTPases in nutrient response. Nat. Cell Biol. 10, 935–945.

Kwiatkowska, K., Sobotka, A., 1999. Signaling pathways in phagocytosis. Bioessays 21, 422–431.

Kops, G.J., de Ruiter, N.D., de Vries-Smits, A.M., Powell, D.R., Bos, J.L., Burgering, B.M., 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398, 630–634.

Leevers, S.J., Weinkove, D., MacDougall, L.K., Hafen, E., Waterfield, M.D., 1996. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. 15, 6584–6594.

Lazaris-Karatzas, A., Montine, K.S., Sonenberg, N., 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature 345, 544–547.

Murray, I., 1971. Paulescu and the isolation of insulin. J. Hist. Med. 26, 150–157.

von Mehring, J., Minkowski, O., 1890. Diabetes mellitus nach Pankreasexstirpation. Arch. Exp. Pathol. Pharmakol 26, 371–387.

Musacchio, A., Cantley, L.C., Harrison, S.C., 1996. Crystal structure of the breakpoint cluster region-homology domain from phosphoinositide 3-kinase P85alpa subunit. Proc. Natl. Acad. Sci. U.S.A. 93, 14373–14378.

Mora, A., Komander, D., van Aalten, D.M., Alessi, D.R., 2004. PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. 15, 161–170.

McKern, N.M., Lawrence, M.C., Streltsov, V.A., Lou, M.Z., Adams, T.E., Lovrecz, G.O., et al., 2006. Structure of the insulin receptor ectodomain reveals a folded-over conformation. Nature 443, 218–221.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., Pandolfi, P.P., 2005. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 121, 179–193.

Manning, B.D., Logsdon, M.N., Lipovsky, A.I., Abbott, D., Kwiatkowski, D.J., Cantley, L.C., 2005. Feedback inhibition of Akt signaling limits the growth of tumors lacking Tsc2. Genes Dev. 19, 1773–1778.

Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., et al., 1992. Wortmannin, a microbial product inhibitor of myosin light chain kinase. J. Biol. Chem. 267, 2157–2164.

Novak-Hofer, I., Thomas, G., 1985. Epidermal growth factor-mediated activation of an S6 kinase in Swiss mouse 3T3 cells. J. Biol. Chem. 260, 10314–10319.

Pearce, L.R., Komander, D., Alessi, D.R., 2010. The nuts and bolts of AGC protein kinases. Nat. Rev. Mol. Cell Biol. 11, 9–22.

Roth, G.J., Stanford, N., Majerus, P.W., 1975. Acetylation of prostaglandin synthase by aspirin. Proc. Natl. Acad. Sci. U.S.A. 72, 3073–3076.

Rudd, E., Schneider, H., 2000. Lymphocyte signaling: Cbl sets the threshold for autoimmunity. Curr. Biol. 10, R344.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., Downward, J., 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370, 527–532.

Russell, R.C., Fang, C., Guan, K.-L., 2011. An emerging role for TOR signaling in mammalian tissue and stem cell physiology. Development 138, 3343–3356.

Ruvinsky, I., Meyuhas, O., 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem. Sci. 31, 342–348.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P., Blenis, J., 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc. Natl. Acad. Sci. U.S.A. 101, 13489–13494.

Saltiel, A.R., Kahn, C.R., 2001. Insulin signaling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.

Smith, C.J., Rubin, C.S., Rosen, O.M., 1980. Insulin-treated 3T3-L1 adipocytes and cell-free extracts derived from them incorporate 32P into ribosomal protein S6. Proc. Natl. Acad. Sci. U.S.A. 77, 2641–2645.

van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., et al., 1997. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science 277, 805–808.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., Sabatini, D.M., 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., Sabatini, D.M., 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 290–303.

Shamji, A.F., Nghiem, P., Schreiber, S.L., 2003. Integration of growth factor and nutrient signaling: implications for cancer biology. Mol. Cell 12, 271–280.

Samuels, Y., Velculescu, V.E., 2004. Oncogenic mutations of PIK3CA in human cancers. Cell Cycle 3, 1221–1224.

Talmage, D.A., Freund, R., Young, A.T., Dahl, J., Dawe, C.J., Benjamin, T.L., 1989. Phosphorylation of middle T by pp6O c-src: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. Cell 59, 55–65.

Traynor Kaplan, A.E., Harris, A.L., Thompson, B.L., Taylor, P., Sklar, L.A., 1988. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. Nature 334, 353–356.

The European Chromosome 16 Tuberous Sclerosis Consortium, 1993. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 75, 1305–1315.

Teis, D., Wunderlich, W., Huber, L.A., 2002. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell 3, 803–814.

Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat. New Biol. 231, 232–235.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., Bilanges, 2010. The emerging mechanism of isoform-specific PI3K signaling. Nat. Rev. Mol. Cell Biol. 11, 329–341.

Voigt, P., Brock, C., Nurnberg, B., Schaefer, M., 2005. Assigning functional domains within the p101 regulatory subunit of phosphoinositide 3-kinase gamma. J. Biol. Chem. 280, 5121–5127.

Valverde, A.M., Lorenzo, M., Pons, S., White, M.F., Benito, M., 1998. Insulin receptor substrate (IRS) proteins IRS-1 and IRS-2 differential signaling in the insulin/insulin-like growth factor-I pathways in fetal brown adipocytes. Mol. Endocrinol. 12, 688–697.

Verdu, J., Buratovich, M.A., Wilder, E.L., Birnbaum, M.J., 1999. Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat. Cell Biol. 1, 500–506.

Whitman, M., Kaplan, D.R., Schaffhausen, B., Cantley, L., Roberts, T.M., 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature 315, 239–242.

Whitman, M., Downes, C.P., Keeler, M., Keller, T., Cantley, L., 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. Nature 332, 644–646.

Walker, E.H., Perisic, O., Ried, C., Stephens, L., Williams, R.L., 1999. Structural insights into phosphoinositide 3-kinase catalysis and signaling. Nature 402, 313–320.

Wymann, M.P., Pirola, L., 1998. Structure and function of phosphoinositide 3-kinases. Biochim. Biophys. Acta 1436, 127–150.

Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Van Haesebroeck, B., Waterfield, M.D., Panayotou, G., 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol. Cell Biol. 16, 1722–1733.

White, M.F., Shoelson, S.E., Keutmann, H., Kahn, C.R., 1988. A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J. Biol. Chem. 263, 2969–2980.

Wilson, J.C., Rochford, J.J., Wolfrum, C., Gray, S.L., Schinner, S., Soos, M.A., Murgatroyd, P.R., Williams, R.M., Acerini, C.L., Dunger, D.B., Barford, D., Umpleby, A.M., Wareham, N.J., Davies, H.A., Schafer, A.J., Stoffel, M., O'Rahilly, S., Barroso, I., 2004. A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science 304, 1325–1328.

Wang, J., Zhong, C., Wang, F., Qu, F., Ding, J., 2013. Crystal structures of S6K1 provide insight into the regulation mechanism of S6K1 by the hydrophobic motif. Biochem. J. 454, 39–47.

Wunderlich, W., Fialka, I., Teis, D., Alpi, A., Pfeifer, A., Parton, R.G., Lottspeich, F., Huber, L.A., 2001. A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J. Cell Biol. 152, 765–776.

Xu, J., Wang, F., Van Keymeulen, A., Herzmark, P., Straight, A., Kelly, K., Takuwa, Y., Sugimoto, N., Mitchison, T., Bourne, H.R., 2003. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell 114, 201–214.

Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G.A., Backer, J.M., 1998. Regulation of the p85/p110 phosphatidylinositol 3′-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Mol. Cell Biol. 18, 1379–1387.

Yang, J., Cron, P., Good, V.M., Thompson, V., Hemmings, B.A., Barford, D., 2002. Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. Nat. Struct. Biol. 9, 940–944.

Yokouchi, M., Wakioka, T., Sakamoto, H., Yasukawa, H., Ohtsuka, S., Sasaki, A., Ohtsubo, M., Valius, M., Inoue, A., Komiya, S., Yoshimura, A., 1999. APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis. Oncogene 18, 759–767.

Yamamoto-Honda, R., Tobe, K., Kaburagi, Y., Ueki, K., Asai, S., Yachi, M., Shirouzu, M., Yodoi, J., Yokoyama, S., et al., 1995. Upstream mechanisms of glycogen synthase activation by insulin and insulin-like growth factor-I. Glycogen synthase activation is antagonized by wortmannin or LY294002 but not by rapamycin or by inhibiting p21ras. J. Biol. Chem. 270, 2724–2729.

CHAPTER

17

TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases

THE TGFβ FAMILY OF GROWTH FACTORS

The receptor serine/threonine protein kinases (Manning et al., 2002) are all dedicated to relaying signals derived from members of the transforming growth factor β family (TGFβ). The first of these to be identified, TGFβ1, emerged as a (oncogenic) transforming factor for mesenchymal cells. Related proteins were revealed through loss-of-function mutation studies in *Drosophila* (Dpp gene) and *Xenopus* (Vg1 gene).

We now know that TGFβ is a member of a family of structurally related proteins, most of which have little or nothing to do with cell transformation. With 42 members in the human genome, seven in *Drosophila*, and four in *Caenorhabditis elegans*, the TGFβ family is one of the most prominent families of first messengers (Shi and Massague, 2003). The mammalian TGFβ family can be divided into a number of subfamilies: TGFβ, BMP, GDF, activin, inhibin, nodal, myostatin, and AMH. These factors may be produced by many cell types (TGFβ), or just a few (myostatin, MSTN). They may be active from the earliest stages of embryo development through adulthood (BMP), or for only very limited periods (AMH) (Massague et al., 2005).

Myostatin and Big Burgers. Myostatin, a member of the TGFβ family, is a negative regulator of muscle growth. It was first identified in null-mutant knockout mice that exhibit a widespread increase in muscle mass due to hyperplasia (fiber number) and hypertrophy (fiber thickness). A 11-bp myostatin deletion in the Belgian Blue and a missense mutation in the Piedmontese cattle breeds in the C-terminal coding region of the myostatin gene (MSTN) are responsible for the double-muscled

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phenotype in these breeds (McPherron and Lee, 1997). The animals are apparently born with a normal phenotype, the so-called double muscling only appearing at about 4–6 weeks. It is by no means rare for the bulls to achieve a weight exceeding 1300 kg. The animals are docile and the beef is said to be delicious. An inherited mutation in the myostatin gene, boosting muscle growth and reducing fat, also occurs in humans. It may come to no surprise that products that claim to regulate myostatin are already used by many athletes and bodybuilders (Figure 17-1).

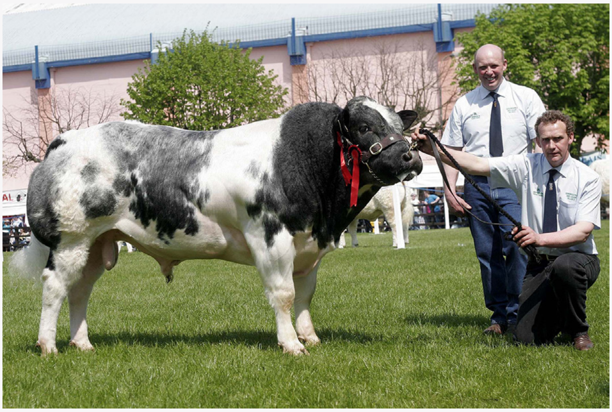

FIGURE 17-1 Big Beef. Springhill Wizzard owned by Martin Brothers, Newtownards, UK, was the best Belgian Blue Junior Bull and Reserve Male Champion at Balmoral Show 2007. Thomas and James Martin are pictured exhibiting the prizewinner. Photograph by kind courtesy of Columba O'Hara and the British Blue Cattle Society.

Several members of the TGFβ family are released as dimers still attached (non-covalently) to their pro-peptides (latency-associated protein, LAP) (Figure 17-2). These latent complexes are often linked to one of the several latent TGFβ-associated proteins (LTBP) (Oklu and Hesketh, 2000). Release of the ligand may occur through proteolytic activity (action of plasmin or the metalloprotease MMP2 or -9) or by interaction with thrombospondin or the integrins αVβ6 and αVβ8 (which modify the pro-peptide by binding to an RGD sequence). The important role of integrin-mediated activation of TGFβ is illustrated by the finding that lack of the RGD motif, and thus failure of the interaction with integrins, results in organ wasting similar to that which occurs in mice that do not express TGFβ1 (Yang et al., 2007; Shull et al., 1992). Stretching the extracellular matrix also releases TGFβ. This occurs by bringing about a conformational change in LAP with the help of the latent-transforming growth factor beta-binding protein 1 (LTBP1). This process is particularly relevant during wound healing

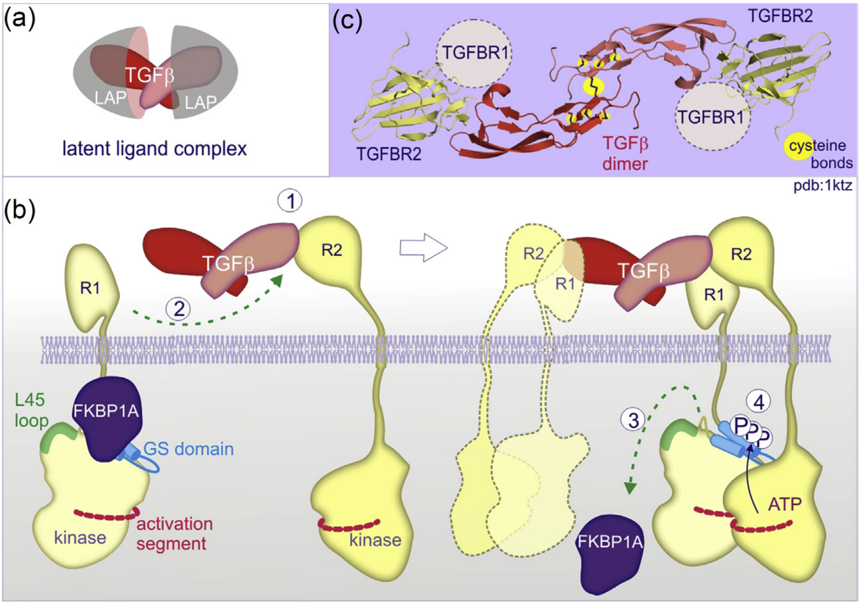

FIGURE 17-2 TGFβ induces receptor dimerization. (a) The TGFβ1-ligand constitutes a dimer which remains associated with its pro-(LAP)-peptides. To bind its receptor, the ligand detaches from the LAP. Different modes of activation not only include degradation by proteases such as plasmin, MMP2, or -9 but also through contact with thrombospondin or the integrins αvβ8 or αvβ8 (vitronectin receptor). Even stretching the extracellular matrix can release TGFβ. (b) TGFβ1 binds to the type-II receptor (1) and allows it to be recognized by the type-I receptor (cooperative binding) (2). The inhibitory FKBP1A detaches (3) and the type-I receptor is phosphorylated by the type-II (4). The activated type-I receptor signals into the cell. Because TGFβ1 is itself a dimer, two receptor complexes can form around the growth factor. The L45 loop is involved in substrate binding. (c) Molecular structure of TGFβ3 associated with two type-II receptors. The presumed orientation of the type-I receptor is indicated. The cysteine knot composed of numerous intramolecular disulfide bonds is indicated in yellow. PDB file; 1KTZ.

where myofibroblasts benefit from the release of TGFβ they themselves bring about (Klingberg et al., 2014). The general idea is of a matrix-associated ligand-reservoir, ready to be activated under favorable conditions.

Sequestration of TGFβ family members also occurs through ligand traps, such as chordin (CHRD), cerberus (CER1), decorin (DCN), follistatins (FSTL1, 3–5), or noggin (NOG) (see Figure 17-9). We return to chordin and noggin, which, by preventing BMP signaling, play an important role in the formation of neuro-ectoderm in *Xenopus laevis*.

TGFβ RECEPTORS, TYPE-I AND TYPE-II

TGFβ1 came to notice in a search for transforming growth factors and initially, it was anticipated that its receptor would be linked, either directly or indirectly, to tyrosine phosphorylations (Roberts et al., 1980). When later, it was found that it inhibits proliferation of epithelial cells, interest shifted to the possibility that it prevents growth factor signaling. Neither of these ideas proved very fruitful. TGFβ1 has no effect on tyrosine phosphorylation, nor (at least when measured on a time scale of minutes) does it affect the early events in EGF or PDGF receptor signaling. So it remained until 1990, when a receptor for activin was cloned and found to contain a putative transmembrane serine/threonine-protein kinase domain (Mathews and Vale, 1991). Similar domains were then found in two of the TGFβ1 receptors (Bassing et al., 1994; Lin et al., 1992; ten Dijke et al., 1994).

Based on their structural and functional properties, the receptors for this family of ligands are divided into subfamilies types I and II (R1 and R2) (Figures 17-2 and 17-4; Luo and Lodish, 1996). They are very similar. Both receptors are glycoproteins. Both have a single membrane span and an intrinsic serine/threonine kinase domain in the C-terminal segment. What distinguishes the type-I receptor is a highly conserved stretch of 30 residues, the GS domain, immediately preceding the kinase domain that regulates R1 kinase activity. Being rather flexible and able to change from firmly attached to a partially detached state, the receptor can switch between inactive and active conformations. The GS domain is normally held firmly attached by the immunophilin FKBP1A (formerly FKBP12) which suppresses any untoward wavering in the activation state of the receptor (Figures 17-2 and 17-4). Phosphorylation of the GS domain by the type-II receptor breaks the inhibitory spell.

Being devoid of the GS domain, the type-II receptor escapes inhibitory control and is therefore constitutively active, always in wait for the approach of the type-I receptor.

The mammalian receptors are homologous with the punt (put), thickveins (tkv), and saxophone (sax) genes of *Drosophila* and with the dauer phenotypes-1 and 4 (daf-1 and daf-4) genes of *C. elegans*. The downstream signaling pathway from the TGFβ1 receptors was revealed by searching for their mammalian homologs of their counterparts in these organisms. It became apparent that the mammalian TGFβ1 receptors transmit signals into cells through the Smad proteins, a unique set of transcription factors (see below) (Liu et al., 1996). Here we outline some of the mechanisms by which members of the TGFβ family of receptors elicit their effects on target cells. We concentrate on the pathway activated by TGFβ1 through the TGFBR1 and TGFBR2 receptors and in particular on the pivotal role of the SMAD proteins in relaying signals to the nucleus. It will become apparent that these pathways are rather similar to those already described for the activation of the STAT transcription factors through the tyrosine kinase-containing receptors such as those for EGF and PDGF and for interferon (return to Chapter 15, "Activating the Adaptive Immune System," Figure 15-9). The main theme is that a receptor complex first recruits and then phosphorylates a transcription factor. This forms an oligomeric complex that translocates to the nucleus to interact

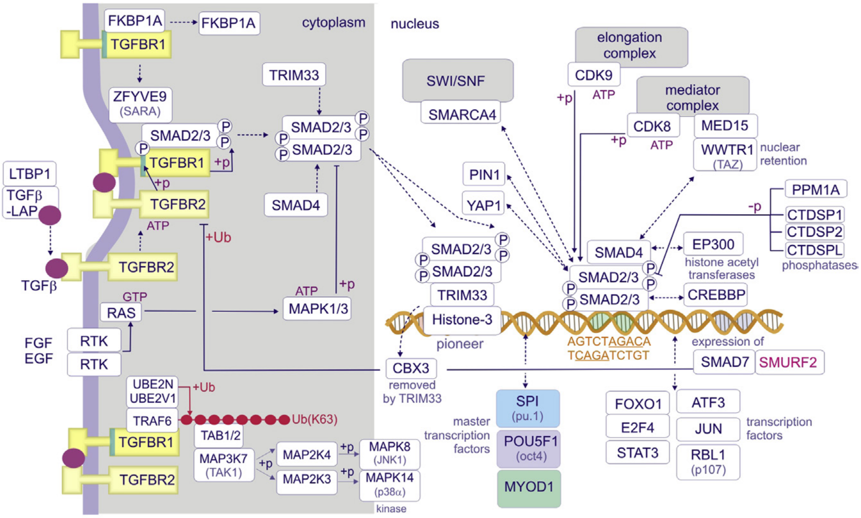

FIGURE 17-3 Overview of TGFβ-mediated signaling events discussed in this chapter.

with DNA-response elements in promoter regions of genes. An overview of the events discussed in this chapter is illustrated in Figure 17-3.

Web resource

You can find the TGFβ pathway on the Kyoto Encyclopedia of Genes and Genomes (KEGG), map No. 04350. URL: www.genome.jp.

TGFβ-MEDIATED RECEPTOR ACTIVATION

TGFβ1 is a disulfide-linked homodimer (Figure 17-2). It gathers pairs of type-I and -II receptors to form heterotetrameric receptor complexes. These may cluster into larger complexes. Ligand-independent homo-oligomers may exist, but do not transmit signals. TGFβ1 can bind to TFGBR2 in the absence of TGFBR1 but not *vice versa*. TGFβ1, however, cannot signal into the cell in the absence of TGFBR1. All this indicates that the most likely sequence of events is that TGFβ1 first binds to the high affinity TGFBR2, thus creating a composite TGFβ/TGFBR2 interface which serves as a binding site for TGFBR1 (process referred to as cooperative binding) (Groppe et al., 2008). In contrast, BMP2 and -4 first bind the type-I receptors and then the type-II. When the ligand brings the two receptors in close proximity, the type-II receptor phosphorylates the serines and threonines in the sequence TTSGSGSGL of the GS domain of TGFBR1 (Figure 17-2(b)).

As with many tyrosine kinase-containing receptors, oligomerization and phosphorylation together constitute the signal for recruitment of effector proteins. However, the receptor serine/threonine kinases employ a quite different approach to the control of kinase activity. On phosphorylation, the GS domain releases the FKBP1A and detaches from the kinase domain. With the loss of these constraints, the kinase becomes catalytically competent (Figure 17-5; Huse et al., 2001). Moreover, the detached phosphorylated GS domain now acts as a docking site for receptor-regulated SMAD proteins, the (unique) substrates of the type-I receptors. This general mechanism applies to all TGFβ and BMP receptors, from *Drosophila* to mammals.

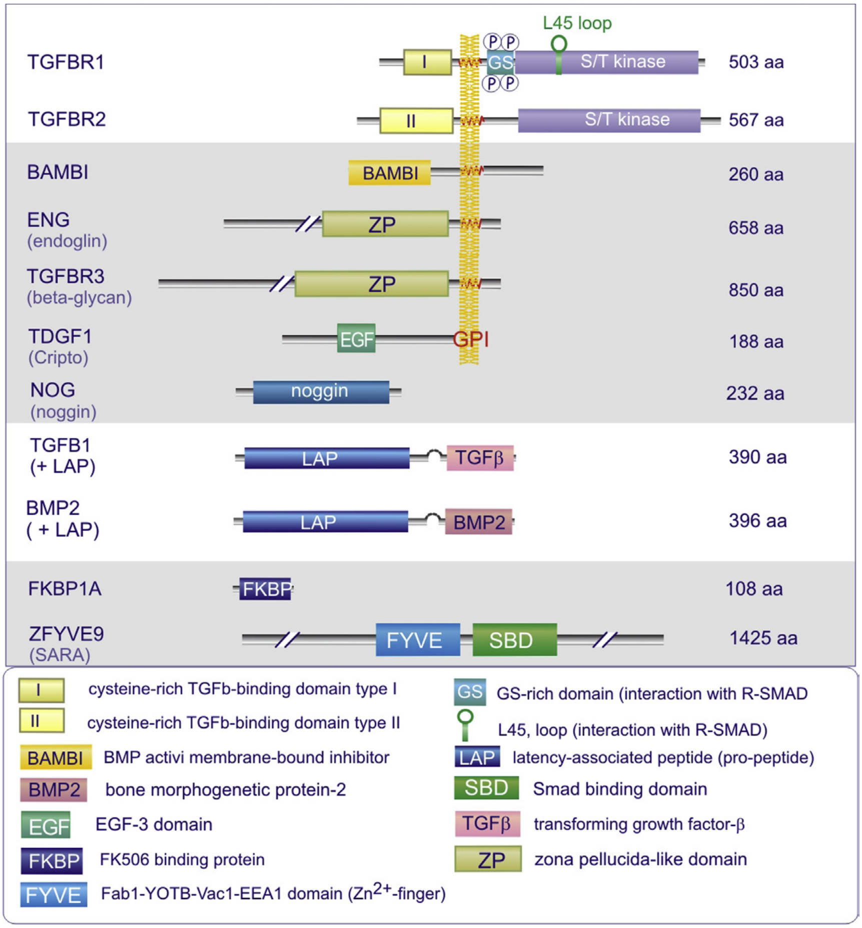
TGFBR1
L45 loop
P P
I
GS
S/T kinase
P P
503 aa
TGFBR2
II
S/T kinase
567 aa
BAMBI
BAMBI
260 aa
ENG
(endoglin)
ZP
658 aa
TGFBR3
(beta-glycan)
ZP
850 aa
TDGF1
(Cripto)
EGF
GPI
188 aa
NOG
(noggin)
noggin
232 aa
TGFB1
(+ LAP)
LAP
TGFβ
390 aa
BMP2
(+ LAP)
LAP
BMP2
396 aa
FKBP1A
FKBP
108 aa
ZFYVE9
(SARA)
FYVE
SBD
1425 aa
I cysteine-rich TGFb-binding domain type I
II cysteine-rich TGFb-binding domain type II
BAMBI BMP activi membrane-bound inhibitor
BMP2 bone morphogenetic protein-2
EGF EGF-3 domain
FKBP FK506 binding protein
FYVE Fab1-YOTB-Vac1-EEA1 domain (Zn2+-finger)
GS GS-rich domain (interaction with R-SMAD
L45, loop (interaction with R-SMAD)
LAP latency-associated peptide (pro-peptide)
SBD Smad binding domain
TGFβ transforming growth factor-β
ZP zona pellucida-like domain

FIGURE 17-4 Domain architecture of TGFβ, its receptors, and associated proteins. Type I and II receptors contain a serine/threonine-protein kinase domain and a cysteine-rich TGFβ-binding domain. The type-I receptor contains an additional GS domain and a number of phosphorylation sites that are involved in its activation by the type-II receptor (which is constitutively activated).

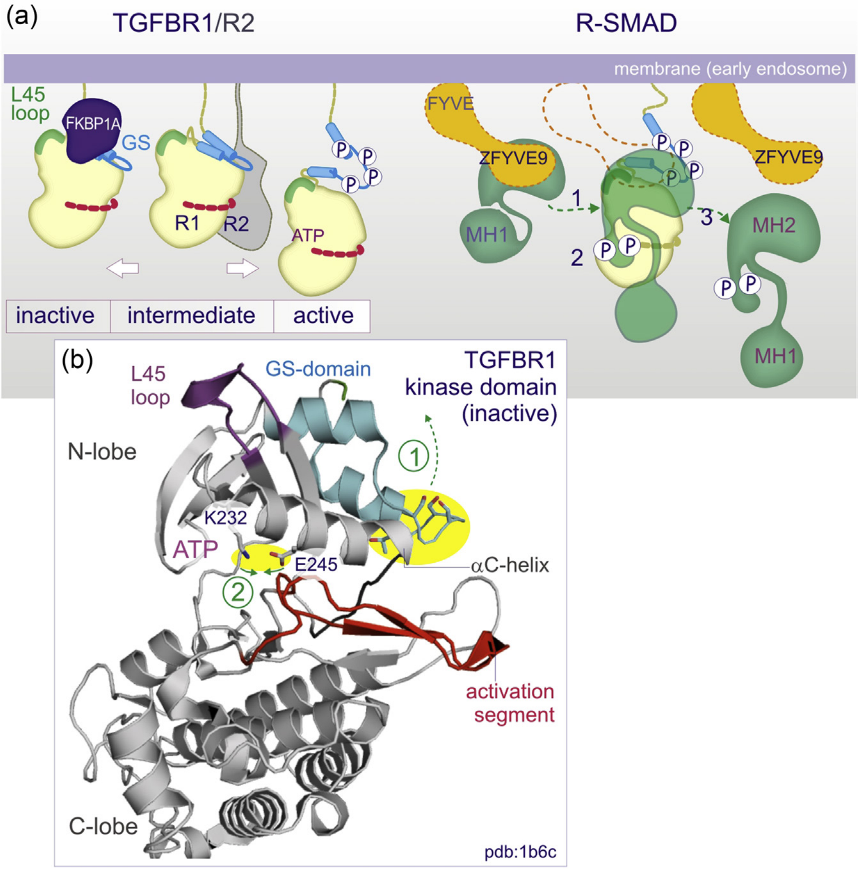

FIGURE 17-5 Activation of the TGFβ receptor and of receptor-regulated Smad proteins. (a) The TGFβ type-I receptor exists in three different states. *Inactive* has the GS wedged into the N-lobe by FKBP1A; *intermediate* allows oscillation between the attached and partly detached positions of the GS; in the *active* state the phosphorylated GS cannot wedge into the N-lobe. Phosphorylation is induced by the close proximity of the types-I and -II receptors. The phosphorylated GS, together with the L45 loop, form the binding site of receptor-regulated SMAD proteins (1), which are subsequently phosphorylated on two serine residues at their C-termini (2). ZFYVE9 facilitates the interaction between receptor-regulated SMAD proteins and the type-1 receptor. The phosphorylated receptor-regulated SMAD detaches from the receptor (3). The events occur at the plasma membrane/early endosome transition. (b) The structure of the TGFβ type-I receptor reveals a typical kinase domain having a small N-terminal and a large C-terminal lobe with a catalytic cleft at the interface. The activation segment (red) is structured but the αC-helix is not oriented to coordinate the binding of ATP. This is illustrated by the large distance between K232 and E245. This is due to interference by the GS domain (light blue) which is wedged into the N-lobe. The threonine and three serine residues phosphorylated in the GS domain are shown as sticks in a yellow circle. In order to achieve catalytic competence the GS domain detaches partially (1) so allowing K232 and E245 to approach each other (2) and to put ATP in place for the transfer of the γ-phosphate onto substrate. The L45 loop, which interacts with the L3 loop of receptor-regulated SMAD proteins is highlighted in purple.

ACCESSORY AND PSEUDO-RECEPTORS: TGFBR3, ENG, TDGF1, AND BAMBI

The quest for cell surface TGFβ-binding proteins revealed a third set of receptors, quite distinct from the TGFBR1 and -R2. Their intracellular domains are devoid of any sequence motif that could be involved in signal transduction. TGFBR3 (formerly betaglycan), endoglin (ENG), and TDGF1 (formerly cripto) are co-receptors that support signaling. BAMBI is a pseudo-receptor that inhibits signaling.

TGFBR3

TGFBR3 is a transmembrane proteoglycan that binds isoforms of TGFβ (Wang et al., 1991). This is vital in the case of TGFβ2 as it facilitates its binding to the receptor, leading to the formation of a ternary complex comprising TGFβ2, TGFBR2, and TGFBR3 (Lopez-Casillas et al., 1991). TGFBR1 then binds to the complex, displacing the TGFBR3. Expression of TGFBR3 in cells normally devoid of this co-receptor causes an increase in TGFβ2 binding and a concomitant increase in sensitivity to the ligand (Figure 17-6; Lopez-Casillas et al., 1993). TGFBR3 also binds inhibin (a dimer of INHA/INHB or INHA/INHBB), promoting its attachment to activin receptors (ACVR2A/B), and then opposing the binding of activin (a dimer of INHBA/INHBA) (Lewis et al., 2000). Inhibins and activins were first identified as factors that, respectively, suppress or stimulate secretion of FSH from pituitary gonadotropes (Figure 17-6(b); Ling et al., 1986). This functional antagonism is explained by the finding that they both bind the activin receptor ACVR2A/B. Binding of activin promotes the recruitment of the activin type-I receptor, ACVR1B, to initiate the signal. Inhibin recruits TGFBR3 to form a stable complex so that there is a loss of the activin signal due to depletion of available ACVR2A/B. This puts a block on further proceedings (Figure 17-6(b); Lewis et al., 2000). Expression of TGFBR3 in cells that normally respond poorly enhances the sensitivity to inhibin.

In vivo, functional role of TGFBR3 (betaglycan) as a co-receptor is vital. Mice lacking betaglycan die during embryogenesis with heart and liver defects (Wiater et al., 2006), and targeting betaglycan during development disrupts mesenchyme formation in the heart and branching morphogenesis in the lung (Brown et al., 1999). In the adult, loss of betaglycan is associated with tumor formation.

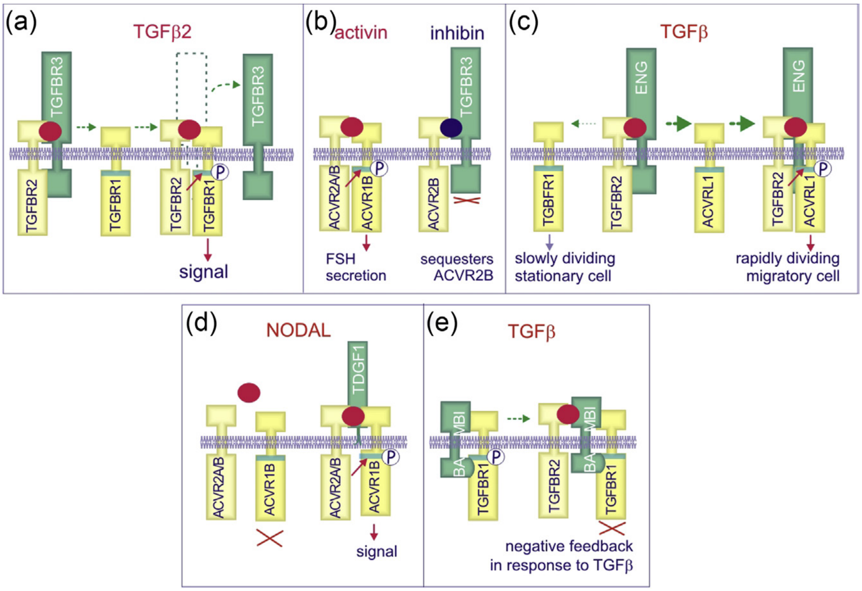

FIGURE 17-6 Accessory and pseudo-receptors. Several membrane proteins bind the TGFβ-family of ligands. None of these has intracellular signaling motifs, instead they re-enforce or inhibit signaling by affecting the interaction between the ligand and its cognate receptors. (a) TGFBR3 (betaglycan) binds TGFβ and facilitates its access to TGFBR2. Once a complex is formed with the TGFBR1, it detaches. TGFBR3 is indispensable for TGFβ2-mediated signaling. (b) Inhibin binds TGFBR3 and ACVR2B and prevents the interaction with ACVR1B resulting in formation of nonfunctional type-II receptors. (c) Endoglin (ENG) facilitates the formation of ACVRL1/TGFBR2 complexes and in endothelial cells it shifts the balance toward an ACVRL1 response (rather than a TGFBR1 response). (d) TDGF1 (Cripto) is bound to the membrane by a glycosyl–phosphatidyl anchor. It binds NODAL attached to ACVR2A/B and facilitates signal propagation through enhanced recruitment of ACVR1B. (e) Expression of BAMBI is induced by TGFβ signaling (negative feedback response). It forms a complex with TGFBR1 and prevents its phosphorylation by TGFBR2 thereby blocking the TGFβ response.

Endoglin

Endoglin (ENG), originally discovered as a glycoprotein-antigen highly expressed on human endothelial cells (Gougos and Letarte, 1988), facilitates the interaction of TGFβ1 already bound to TGFBR2 with ACVRL1 (not the classic type-I TGFB receptor) in endothelial cells (Cheifetz et al., 1992). Thus it determines the balance between ACVRL1 signals, leading to proliferation and migration of endothelial cells, and TGFBR1 signals that inhibit proliferation and migration (Figure 17-6(c)). Since both receptors are expressed on endothelial cells, the presence of endoglin decides the outcome of the response to TGFβ, favoring an ACVRL1-mediated proliferative and invasive response. It facilitates the formation of new blood vessels (angiogenesis) (Goumans et al., 2003; Lebrin et al., 2004). Inherited absence of functional endoglin is associated with the disorder hemorrhagic telangiectasia type-I (HHT1), characterized by a leaky and poorly developed vascular bed (McAllister et al., 1994).

TDGF1 and TDGF1P3

TDGF1 (formerly Cripto) is a small extrinsic membrane protein of the EGF-CFC gene family, members of which take part in embryonic anterior–posterior axis development (Shen and Schier, 2000). It binds to NODAL, promoting the formation of active receptor dimers (ACVR1B/C with ACVR2A/B). TDGF1P3 (cripto3) binds activin, when attached to ACVR2A/B, but here it impedes access to the type-I receptor. A similar inhibitory action has been shown for TGFβ (not shown). Its tumor-promoting action, TDGF stands for teratocarcinoma-derived growth factor, may in part be explained by its capacity to block activin- or TGFβ-mediated growth inhibition of epithelial cells (Gray et al., 2006) (Figure 17-6(d)).

BAMBI

BAMBI (BMP and activin membrane-bound inhibitor) associates with cytoplasmic domain of TGFBR1 receptors in the absence of ligand (Figure 17-6(e)). In so doing, it prevents phosphorylation by TGFBR2 and inhibits signaling from activin, BMP, and TGFβ (Onichtchouk et al., 1999).

DOWNSTREAM SIGNALING: *DROSOPHILA*, *CAENORHABDITIS*, AND SMAD

Genetic screening of accessible organisms such as *Drosophila* and *C. elegans* has provided pointers to the mechanism of TGFβ signaling in mammalian cells. The gene, decapentaplegic (Dpp), when mutated, causes pattern deficiencies and duplications in structures derived from one or more of the 15 major imaginal disks in *Drosophila*. Later, with the appearance of the segments, Dpp functions in the definition of boundaries between the segmental compartments. As part of this process, Dpp defines the position of the future limbs, wings, legs, and antennae. It also has a role in the structuring of the mesoderm.

The Dpp gene product acts through three receptors that are homologous to the family of mammalian TGFβ receptors: thick veins and saxophone resemble TGFBR1, and punt resembles TGFBR2. Genetic screening revealed a mutation that, when combined with Dpp mutations associated with a feeble phenotype, generates one that is more severely affected. The mutant gene so obtained, Mad, yields flies that exhibit defects resembling those due to mutated Dpp (Raftery et al., 1995). However, since wild-type Dpp cannot restore the defects induced by mutations in Mad, Dpp must lie upstream of Mad (Figure 17-7; Newfeld et al., 1996).

- Decapentaplegic, paralyzed at 15 sites.
- Imaginal, from imago, the final and perfect stage or form of an insect after its metamorphoses.
- Receptor regulated: The *Drosophila* homologs are MAD and dSmad2, the *C. elegans* homologs are Sma-2 and -3.
- Common mediator: The *Drosophila* and *C. elegans* homologs are Medea and Sma-4, respectively.

Web resource

For more information about *Drosophila* and its genes we recommend “the interactive fly,” URL: www.sdbonline.org/fly/aimain/1aahome.htm.

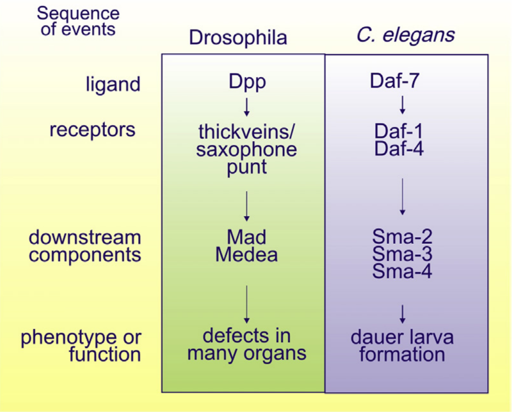

FIGURE 17-7 Signal transduction pathways downstream of serine/threonine kinase receptors in two phyla. Homologies between the genes implicated in these pathways allowed the elucidation of the sequence of events downstream of the TGFβ receptor in mammals.

The nematode *C. elegans* responds to conditions of overcrowding and starvation by developmental arrest as a dauer (resilient, durable) larva. Genetic screening of mutants having this phenotype revealed a number of genes (daf-1, -2, -3, -4, etc.) of which daf-1 and daf-4 code for serine/threonine-receptor protein kinases (Estevez et al., 1993; Georgi et al., 1990). Daf-4 mutants are dauer-constitutive and the larvae are smaller than the wild types. Screening for mutants with similar phenotypes revealed three more genes, sma-2, -3, and -4 (Savage et al., 1996) that act downstream of Daf-4. Mad (fly) and Sma (worm) proteins are homologous, and with the sequences to hand, eight human homologs coding for SMAD proteins were revealed (Liu et al., 1996; Heldin et al., 1997).

SMAD PROTEINS HAVE MULTIPLE ROLES IN SIGNAL TRANSDUCTION

The SMAD proteins have two regions of homology, MH1 and MH2, connected by a more divergent linker segment (Figure 17-8). The N-terminal MH1, highly conserved in all SMAD proteins (except 6 and 7), binds to DNA at the SMAD-binding element (though curiously, the most abundant splice variant of SMAD2 carries an insert that blocks binding to DNA). MH1 also interacts with transcription factors such as JUN, ATF3, SP1, and RUNX1. The linker constitutes a flexible segment that forms a hotspot of phosphorylation sites (example shown for human SMAD1) and operates in the integration of several signaling pathways. The regulated SMAD proteins carry ubiquitinylation sites. They also contains a PPxY motif that acts as a binding site for WW-domain-containing proteins (E3-ubiquitin ligases SMURF1 and NEDD4L, transcription factor YAP1 and the co-activator SIN1). In SMAD4, the linker region contains a nuclear export signal (NES) (Figure 17-8).

The MH2 domain, a particularly versatile protein-interacting module, is conserved in all SMAD proteins. A set of adjoining hydrophobic patches (the hydrophobic corridor) mediates interactions with cytoplasmic retention proteins such as ZFYVE9 (SARA), the nuclear pore protein NUP214, and with a number of transcription co-activators/co-repressors. The MH2 domains of SMAD1, -2, -3, and -5 carry a conserved C-terminal SxS motif, the substrate of type-I receptors (TGFBR1, ACVR1B, etc). When phosphorylated, this interacts with a basic pocket in MH2 to provoke the assembly of hetero-oligomeric complexes (see below).

SMAD proteins have different functions, controlled through their selective interactions with type-I receptors and with each other (Kretzschmar and Massague, 1998; Figures 17-5 and 17-10). On the basis of their structures and functions they are divided into three groups: (1) receptor-regulated proteins, SMAD1, -2, -3, and -5; (2) common mediator SMAD4; and (3) inhibitory proteins SMAD6 and -7 (Figure 17-8). Below we elaborate further on their structure and function.

Receptor-regulated SMAD proteins (1, 2, 3, and 5)

Receptor recognition

The receptor-regulated SMAD proteins are phosphorylated by the activated TGFBR1. Short structural elements in TGFBR1 and in the SMAD proteins determine the specificity of interaction, the exposed L45 loop in the type-I receptor kinase domain (Figure 17-5) interacting with the L3 loop

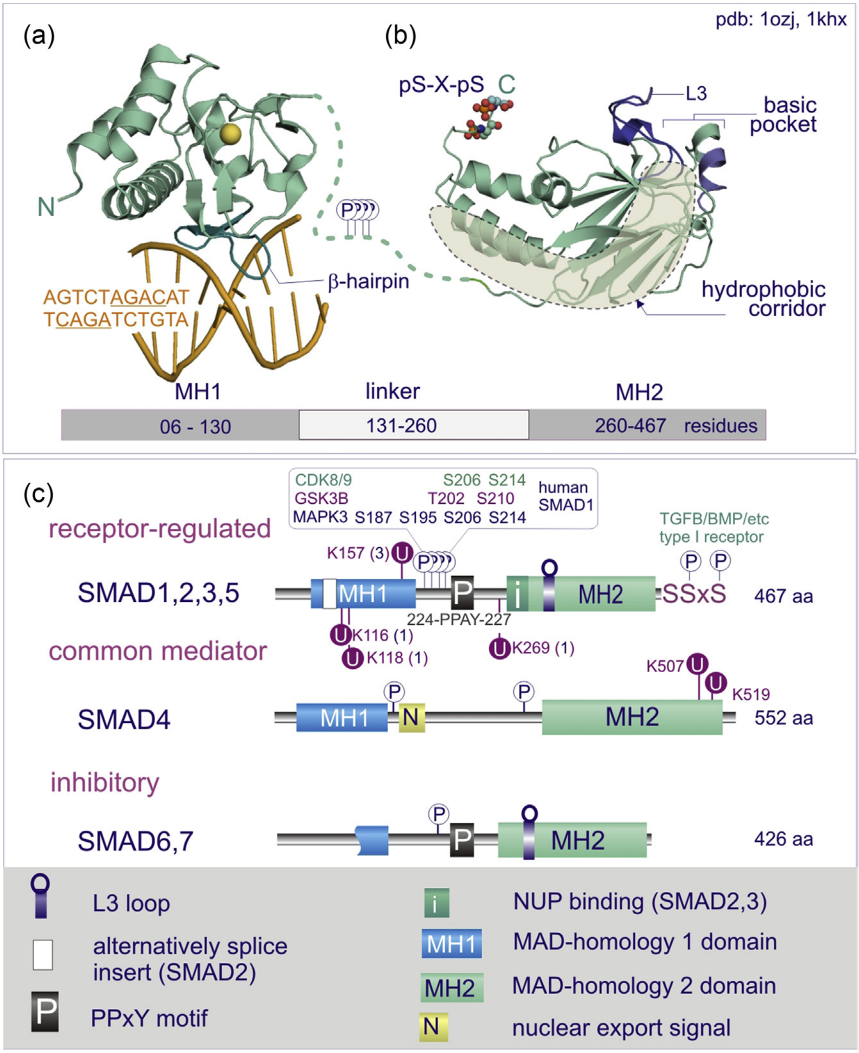

FIGURE 17-8 Molecular structure and domain architecture of Smad proteins. (a) Receptor-regulated SMAD proteins have three distinct segments, a conserved MH1 domain, a more divergent linker region, and a highly conserved MH2 domain. MH1 is stabilized by Zn^{2+} (yellow sphere) and contains a β-hairpin that binds the SMAD-binding element (SBE). (b) The MH2 domain contains a C-terminal SxS-motif that is phosphorylated by the type-I receptor. It also has an L3 loop and basic pocket, which interact with L45 and the phosphorylated GS domain of the receptor. It exposes a hydrophobic corridor which is the site of attachment of numerous proteins among which is ZFYVE9. It also interacts with numerous other components, including TGFBR1, nuclear pore proteins, co-activators and co-repressors, and DNA-binding cofactors. It is involved in SMAD oligomerization. (c) SMAD transcription factors are subdivided in three groups based on functional and structural criteria. Receptor-regulated SMAD proteins contain the SxS motif which is phosphorylated by type-I receptors. They contain an NUP-binding motif as well as a PPxY motif with which they interact with the E3-ubiquitin ligase SMURF1 or NEDD4L. The common-mediator SMAD4 forms complexes with all receptor-regulated SMAD proteins interacting with their phosphorylated SxS motif. It serves to stabilize the complex. It lacks the L3 loop and does not interact with type-1 receptors. SMAD6 and -7 lack a functional MH1 domain as well as the SxS motif. They prevent TGFβ signaling. They too bind SMURF1 through a PPxY motif in the linker region. All SMAD proteins contain numerous sites that are phosphorylated by serine/threonine protein kinases (just a few are indicated). Receptor-regulated SMAD proteins carry ubiquitinylation sites. These phospho-residues act as binding sites for cofactors and for E3-ubiquitin protein ligases and thus play an important part in the regulation of gene expression as well as protein half-life.

in the MH2 domain of the SMAD (Figure 17-8). By exchanging selected residues in these domains it is possible to switch the signaling specificity of the TGFβ and BMP pathways (Chen et al., 1998; Feng and Derynck, 1997). Thus, TGFBR1, ACVR1B, and ACVR1C recognize SMAD2 and -3, whereas ACVR1, ACVRL1, BMPR1A, and BMPR1B recognize SMAD1, -5, and -8 (summarized in Figure 17-9). In this way, different ligands, binding different receptor combinations, provide different signals through the recruitment of specific combinations of SMAD proteins. Note that TGFβ can activate both pathways, depending on the receptor combination and the presence of endoglin (Wu and Hill, 2009; Lebrin et al., 2004).

The MH2-domain qualifies as a phosphoserine-binding domain and offers yet another means by which receptor signaling complexes are formed (similitude with SH2 and PTB domains which recognize phosphotyrosine-containing sequences). Structurally, it shares striking homology with the forkhead-associated domain (FHA) but not with 14-3-3, both of which also recognize phosphoserine/threonine residues (Durocher et al., 2000).

Whereas the interaction between the loops, L45 and L3, determines specificity, phosphorylation of the C-terminal SxS motif acts as the on/off signal, roughly equivalent to phosphotyrosine binding to SH2- or PTB-domain-containing proteins (return to Chapter 10 "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Section "Protein Domains that Bind Phosphotyrosines and the Assembly of Signaling Complexes"). The phosphorylated SxS motif is recognized by a basic pocket in the SMAD proteins, adjacent to the L3 loop (Figure 17-8). Serine phosphorylation of the SxS motif in the MH2 domain causes a structural alteration that decreases affinity of the SMAD proteins for cytoplasmic anchors, increases their affinity for nuclear pore proteins, and allows them to bind each other and to SMAD4 (Figure 17-10).

Cytoplasmic retention of receptor-regulated SMAD proteins

In their basal steady state, the receptor-regulated SMAD proteins are mainly present in the cytoplasm (Pierreux et al., 2000). A number of proteins function as anchors. Examples are Disabled-2, AXIN, cPML, ZFYVE9 (formerly SARA) (all of which bind Smad-2 and -3), and ELF (SMAD-3 and -4). TRAP-1 and TLP bind to SMAD4 but only after treatment with TGFβ and it is not sure whether they play a role in cytosolic retention in "resting" cells (Massague et al., 2005). The best characterized of these is ZFYVE9 (Tsukazaki et al., 1998), originally identified as an endosomal protein (return to Figure 17-5(a)).

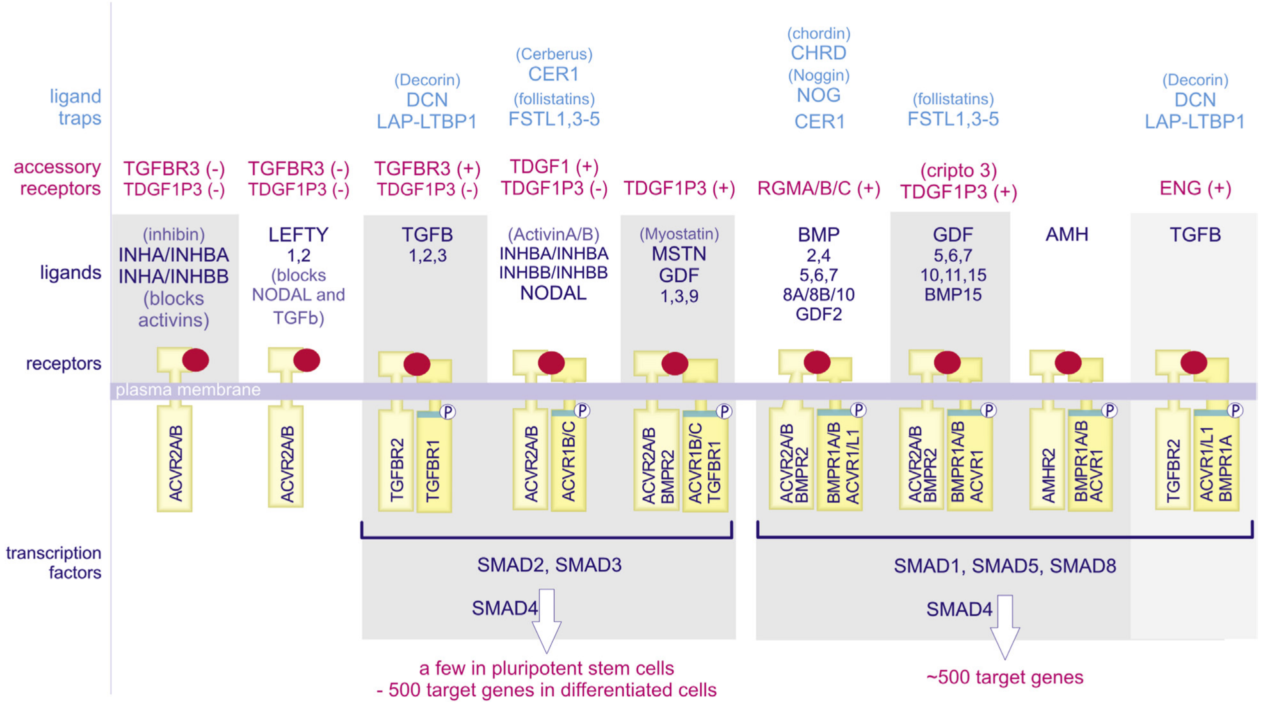

FIGURE 17-9 The TGFβ-family of ligands, their traps, and their ligands. Ligand traps prevent access to the receptor. Accessory receptors facilitate ligand binding and receptor complex formation. The different receptor combinations can roughly be divided in two groups: the first group ("TGFβ/activins") recruits Smad2 and -3 whereas the second group ("BMP") recruits Smad1, -5, and -8. The type-II receptors phosphorylate and activate the type-I receptors. *Information from Ref.* Moustakas and Heldin (2009).

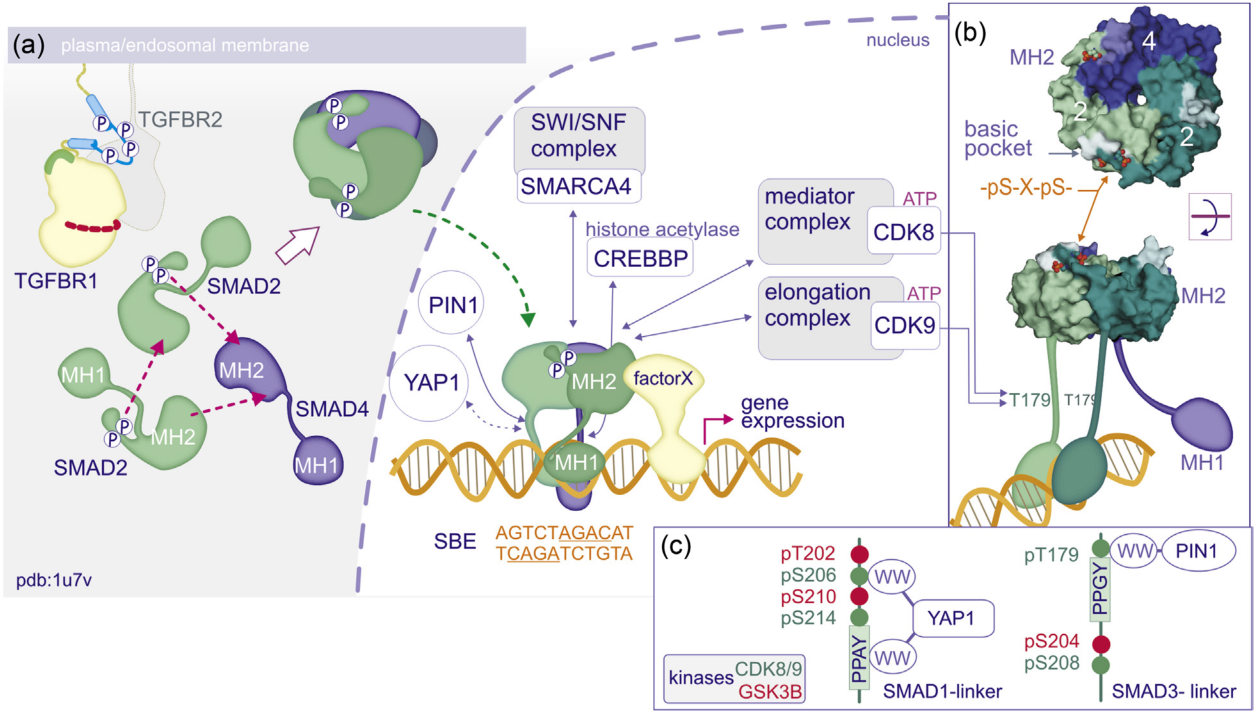

FIGURE 17-10 SMAD activation and nuclear translocation. (a) On phosphorylation of the C-terminal SxS-motif, receptor-regulated SMAD proteins complex with each other and then with SMAD4. The phosphoserines bind the basic pocket in the MH2 domain. The trimers enter the nucleus to bind DNA at the SMAD-binding element (SBE). The complexes also bind other DNA-binding proteins and transcriptional co-factors. (b) Detail of a SMAD2/SMAD2/SMAD4 complex showing the interaction of the phosphoserines in the C-terminus with the basic pocket of the MH2 domain. The SMAD proteins are viewed from two different angles to show the presumed orientation of the MH2 relative to the DNA-binding MH1 domain. Two MH1 domains, in inverse orientation, can interact with the DNA enhancer element (underlined nucleotides in panel a). (c) Phosphorylation sites in the linker region, combined with an adjacent PPxY motif, serve as binding sites for WW-domain-containing proteins. A first set of phosphorylations occurs through CDK8 and CDK9, protein kinases that take part in the mediator- and elongation complex, respectively. Depending on the SMAD, the linker region either recruits a small peptidyl–prolyl *cis–trans* isomerase, PIN1 (for SMAD3) or the AP-1-like transcription factor YAP1 (for SMAD1). *(The phosphorylation scheme is adapted from Aragón et al. (2011).)* We return to the GSK3B phosphorylation sites in Figure 17-13.

ZFYVE9, a Zinc finger and FYVE-domain-containing protein, operates in two ways. First, it binds the MH2 domain in the hydrophobic corridor that also binds the nuclear transport proteins (Wu et al., 2000). Phosphorylation of the SxS motif disrupts the binding with ZFYVE9 and this may constitute the nuclear translocation signal. Secondly, the FYVE domain binds to PIP-3, abundant in early endosomal vesicles (Misra and Hurley, 1999; Di Guglielmo et al., 2003). It has been suggested that effective phosphorylation of SMAD2 and -3 only occurs by receptors present in the early endosomal compartment. However, with all this said, there is no genetic evidence to support the idea that ZFYVE9 is directly involved in signaling through SMAD2 or -3 (Massague et al., 2005) and, importantly, there is no interaction between SMAD1 and ZFYVE9, so it is not clear if a similar protein is involved in signaling from BMP receptors. For more information about PIP3 return to Chapter 16, "Signaling through the Insulin Receptor," Section "Signaling through phosphoinositides."

Common-mediator SMAD4

SMAD4 does not interact with the receptors and it lacks the C-terminal SxS phosphorylation motif though it does form hetero-oligomer complexes with the receptor-regulated SMAD proteins (Figure 17-10). In mammalian cells it binds to phosphorylated SMAD1, -2, -3, and -5 forming complexes that transfer to the nucleus, there to act as transcription factors. It plays an important role in the interaction with the histone acetylation enzyme EP300 (Janknecht et al., 1998).

It is not certain whether SMAD4 links with other SMAD proteins in the cytoplasm or the nucleus. For sure it does not interact with ZFYVE9 and no cytosolic retention proteins have been described in resting cells, but it does carry a NES which interacts with CRM1, a nuclear export receptor. In this way SMAD4 is retained in the cytosol unless it teams up with phosphorylated receptor-regulated SMAD proteins or with other proteins that mask the NES (Inman et al., 2002).

SMAD–SMAD complexes

The C-terminal SxS phosphorylation motif of the receptor-regulated SMAD proteins resembles the phosphorylation motif of the GS domain in the type-I receptors. Following phosphorylation, the basic pocket in the MH2 domain turns its attention to fellow SMAD proteins to form hetero-oligomeric complexes (Figure 17-10; Wu et al., 2001; Qin et al., 2001). The stoichiometry of the hetero-oligomer complexes remains a matter of some debate but heterotrimers, comprising two receptor-regulated SMAD proteins and one SMAD4 seem to be prevalent (Chacko et al., 2004).

Nuclear import and export

Migration of the individual SMAD proteins into the nucleus occurs at all times and either involves nuclear importins or not. The hydrophobic corridor of the MH2 domain interacts directly with the FG-repeat region on nucleoporins (for instance NUP214, Figure 17-15), in this way obviating the nuclear transport receptors (importins α and β) (Xu et al., 2002). Kinetic analysis has demonstrated that TGFβ-signaling minimally alters nuclear import rates of SMAD complexes but substantially decreases the rate of export (Schmierer and Hill, 2005). Nuclear retention is thought to be mediated by the compendium of nuclear-binding partners (see below) of which WWTR1 (formerly TAZ) is crucial (Varelas et al., 2008). WWTR1 is associated with MED15 (formerly ARC105), a component of the mediator complex. We note that the nuclear localization of WWTR1 is under control of phosphorylation by the protein kinase complex LATS1-MOB1 which operates in the Hippo pathway (Hong and Guan, 2012). Phosphorylation renders WWTR1 inactive through cytoplasmic retention (sequestered by 14-4-3 protein). In epithelia, the Hippo pathway initiates at the tight junctions and has a general “tumor suppressor” action (promoting tissue integrity) (Ramos and Camargo, 2012). By preventing nuclear retention of SMAD complexes, the Hippo pathway opposes the TGFβ-mediated epithelial–mesenchymal transformation (discussed at the end of the chapter).

REGULATION OF TRANSCRIPTION BY SMAD PROTEINS

Activation of gene expression

SMAD heterotrimers bind to DNA through their MH1 domains. It is not clear how the three domains participate in DNA binding. A structural analysis has shown that only two MH2 domain can bind a single enhancer element (sequence AGAC) and these are most likely those of the receptor-regulated SMADS (Chai et al., 2003). SMAD4 is not obligatory for TGFβ signaling but it is required to provide the highest response and this is readily explained by the fact that SMAD4 stabilizes SMAD–DNA-binding complexes and also participates in the recruitment of co-activators. To achieve high affinity and selectivity, the SMAD complex interacts with other DNA-binding factors (Seoane et al., 2004) (represented by “factor X” in Figure 17-10). This suggests that it is not the DNA contact that provides selectivity for gene targeting between the different SMAD proteins (Shi et al., 1998). Rather, the combinations of the SMADS with their partners provide the necessary levels of specificity. Several cofactors have been identified, belonging to different families of DNA-binding proteins that account for the great breadth of TGFβ transcriptional responses. As we discuss below, Section “Cooperation with other pathways and other transcription factors," master transcription factors are essential partners (Mullen et al., 2011).

In addition to all that has just been related, the heterotrimeric Smad complexes also interact with other DNA-binding proteins and transcriptional cofactors to form even bigger aggregates. The linker-region of SMAD4 recruits EP300 (Janknecht et al., 1998; de Caestecker et al., 2000) and the M1-region of SMAD2 (or -3) recruits CREBBP (Chen et al., 2007), thus bringing histone acetyltransferases into the gene-enhancer region. In addition, SMAD2 recruits the mediator and elongation complex (Kato et al., 2002) as well as the ATP-dependent nucleosome remodeling complex SWI/SNF. The latter complex, composed of ARID1A, ARID2, SMARCC1, and SMARCC2 is recruited through SMAD2-mediated binding of SMARCA4 (SWI/SNF-related matrix-associated regulator of chromatin, formerly known as BRG1) (Ross et al., 2006). Acetylation of histone-3, in particular at Lys-9 (K9) and -18 (K18), combined with the action of SWI/SNF, brings about a loose nucleosome configuration which allows binding of other regulatory transcription factors and importantly paves the way for the action of RNA-polymerase II (POLR2A). The mediator complex plays a role in the positioning of the pre-initiation transcription complex, comprising the TFII-transcription factors and the RNA-polymerase II. The elongation complex, as the name indicates, regulates elongation of the RNA-polymerase II (Shilatifard et al., 2003).

The mediator and elongation complex are associated with cyclin-dependent protein kinases, CDK8 and -9, respectively. These phosphorylate the linker region of regulated-SMAD proteins. Figures 17-8 and 17-10 illustrate the phosphorylation sites on SMAD1 ("BMP pathway") and on SMAD3 (mainly "TGFβ pathway"). Phosphoserines/threonines in combination with the proximal PPxY motif serve as binding sites for WW-domain-containing proteins (Bork and Sudol, 1994). Among these are YAP1, an AP-1-like transcription factor, and PIN1, a peptidyl-prolyl *cis-trans* isomerase (of which we have no understanding of how it may affect transcription) (Alarcón et al., 2009; Aragon et al., 2012). The action of CDK8/9 is counteracted by the polymerase-II-associated phosphatases CTDSP1, CTDSP2, and CTDSPL (C-terminal domain-associated small phosphatase). Apart from their role in the TGFβ pathway, these phosphatases play a fundamental role in the regulation of transcription. Phosphorylation of serine-2 in the so-called heptapeptide repeats (-Y**pS**PTSPS-) of the C-terminal domain of polymerase II (POLR2A) causes a release from promotor-proximal pausing. The phosphatases block elongation whereas protein kinases, such as CDK9, promote elongation (Cheng and Price, 2007). Thus, kinases and phosphatases that enable/disable polymerase activity simultaneously enable/disable the action of the SMAD proteins. For a brief overview of transcription regulation, return to Chapter 7, "Bringing the Signal into the Nucleus," Section "Transcription and transcription factors."

Repression of gene expression

About a quarter of all TGFβ responses in mammalian cells involve gene repression (Valcourt et al., 2005). Repressive complexes act in different ways. The SMAD complex recruits RBL1 (formerly p107), a protein that resembles the retinoblastoma tumor suppressor. It recruits histone methyltransferases (SUV420H1 and -H2) to the promoter and these stop elongation of the RNA-polymerase through trimethylation of lysine-20 on histone-4 (H4K20Me3) (Kapoor-Vazirani et al., 2011; Jones and Takai, 2001). SMAD2 or -3 also bind TGIF1, a DNA-binding protein that recruits CTBP1 to the DNA. This in turn introduces the histone deacetylation enzyme HDAC1 and causes deacetylation of histone in the enhancer/promoter region. The ensuing tight nucleosome configuration hinders access of regulatory transcription factors and renders elongation of the RNA-polymerase II almost impossible (Melhuish and Wotton, 2000).

Individual SMAD proteins also complex with LEF1/β-catenin or TRIM33

SMAD2, -3, and -4 proteins also combine to form complexes with other DNA-binding proteins. Quite independently of TGFβ signaling, SMAD4 forms a complex with LEF1 bound to β-catenin (CTNNB1) to induce expression of MYC (Lim and Hoffmann, 2006; Figure 17-18). SMAD4 without other SMAD proteins also plays a role in expression of the tight junction protein claudin-1(CLDN1) (Shiou et al., 2007).

SMAD2 and -3 bind TRIM33 (also known as TIF1γ) in competition with SMAD4 (He et al., 2006). This alternative complex comes into action during TGFβ-mediated differentiation of hematopoietic progenitors into red blood cells, acting in parallel with the canonical transcription complex previously described (Xi et al., 2011; He et al., 2006). The SMAD2/3/TRIM33 complex is different from the transcription factor complexes dealt with so far in that it does not bind DNA but methylated and acetylated histone instead. TRIM33 possesses a PHD and bromo-domain that act as readers of the chromatin code (histone modifications). What this means is that SMAD-proteins get access to chromatin regions that normally are not accessible for DNA-binding. Particularly concerned are enhancer regions with the following histone-3 modifications: H3K4 unmodified, triple methylated lysine-9 (H3K9me3), and acetylated lysine-18 (H3K18Ac). These modifications are typical of euchromatin regions in which gene expression is repressed but can be readily activated (poised state) (return to Chapter 7, “Bringing the Signal into the Nucleus,” Figure 7-11) (Lee and Young, 2013; Xi et al., 2011). These regions also contain members of the CBX family of chromatin-binding proteins (formerly known as HP1).

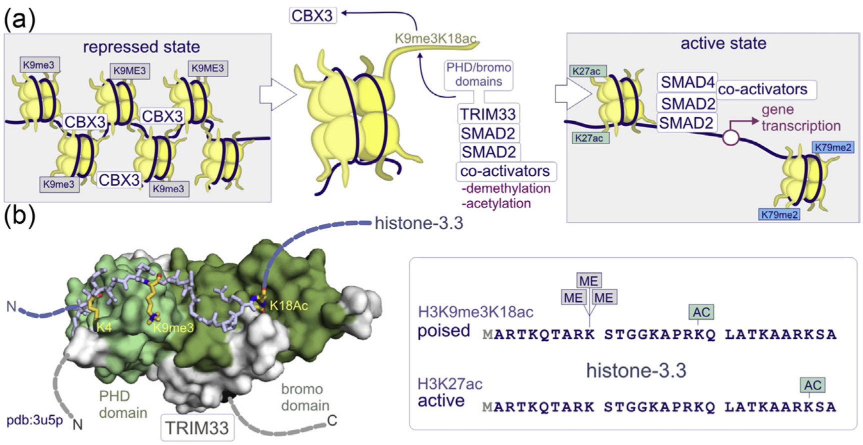

FIGURE 17-11 TRIM33-mediated transcriptional regulation. (a) Certain regions of DNA of the undifferentiated hematopoietic progenitor cells are not transcribed because they are not accessible for regulatory transcription factors. The repressed state is recognized by methylation of lysine-9 in histone-3 (H3K9me3) which attracts CBX3 and leads to compaction of the nucleosomes. The TRIM33 competes with CBX3 for binding to the N-terminal tail of histone-3. Its combined PHD- and bromo-domain has good affinity for triple-methylated lysine-9 and acetylated lysine-18 (H3K9me18ac). When it forms a complex with SMAD2, they remove CBX3 from the nucleosome and they recruit co-activators that further modify histones (demethylation, acetylation) in the enhancer region. This paves the way for SMAD3/SMAD4 complexes and sets in train a change in gene transcription that ultimately results in cell differentiation. (b) Detail of the PHD- and bromo-domain of TRIM33. Not the presence of unmodified K4, triple-methylated K9, and acetylated K18. The sequence indicates the modification sites on histone 3.3. H3K9me3K18ac is a hallmark of a poised enhancer-region, where transcription is repressed but the region remains responsive to regulatory transcription factors. Active enhancer regions are characterized by acetylation of lysine-27 (H3K7ac).

CBX possess two chromo-domains with which they interact with methylated lysine-9 (K9me3) of histone-3. Their presence causes compaction of nucleosomes.

TRIM33 competes with CBX3 (formerly HP1γ) for binding to histone-3 because its PHD domain too interacts with triple methylated lysine-9 (H3K9Me3) (Figure 17-11(b)). Displacement of CBX3 loosens the nucleosomes whereas the SMAD-mediated recruitment of chromatin remodeling enzymes next creates accessible enhancer elements for the SMAD2/3/4 complex (right panel of Figure 17-11(a)). The tandem action causes expression of genes that bring about a change in the transcriptome, made apparent by inhibition of proliferation and the onset of cell differentiation. The SMAD/TRIM33 complex behaves like a pioneer factor (Iwafuchi-Doi and Zaret, 2014). We return to this alternative SMAD complex because it also plays a role in attenuating the TGFβ response (but at a later stage).

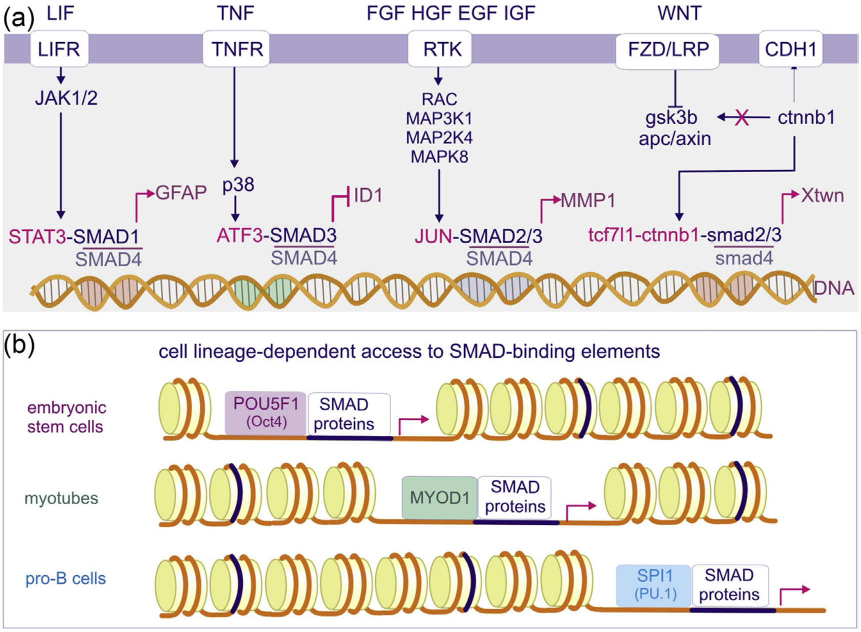

FIGURE 17-12 SMAD proteins cooperate with different pathways and different transcription factors. (a) Through interaction with different DNA-binding transcription factors, SMAD proteins form nodes of integration giving rise to different signal transduction pathways. *(Image adapted from Ref. Massague et al. (2005).)* For the description of events see the text. (b) With respect to the set of genes induced by TGFβ, in a number of cell lines it was shown that this depends on the expression of master transcription factors. These occupy a large number of enhancer elements and determine the global transcriptome of any given cell (determining cell lineage identity). In embryonic stem cells SMAD3 was found associated with the same enhancer regions that bind POU5F1 (formerly Oct4), in myotubes the same enhancer elements that bind MYOD1 also bind SMAD3 and in Pro-B cells it co-localizes with SPI1 (formerly PU.1). *(Image adapted from Ref. Mullen et al. (2011).)*

COOPERATION WITH OTHER PATHWAYS AND OTHER TRANSCRIPTION FACTORS

Pathway cross-talk

Through their cooperativity and the multitude of their interactions, the SMAD proteins provide a versatile platform that provides many forms of cross-talk between signaling pathways. Some of these are illustrated in Figure 17-12(a) and in the following paragraphs.

- The activation of STAT3 (return to Chapter 15, "Activating the Adaptive Immune System" Section "Interferon-α and STAT proteins") by leukemia inhibitor factor (LIF) enforces BMP-induced expression of GFAP. This way, STAT3 and SMAD1 work together to drive differentiation of neural stem cells into astrocytes.

- p38 (MAPK14) and JNK1 (MAPK8) (return to Figure 10-27, Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases”) contribute to TGFβ signaling through activation of ATF3 and JUN, both of which interact with SMAD proteins. ATF3 and SMAD3 act together to repress expression of ID1 (Kang et al., 2003). In epithelial cells this is necessary for effective arrest of the cell cycle. Thus TGFβ and TNF act in concert to slow down the cell cycle, reducing DNA replication and therefore protecting the organism against DNA-damaging agents.
- JUN, as part of the AP1 complex (return to Figure 9-10, Chapter 9, “Protein Kinase C in Oncogenic Transformation and Cell Polarity”) aids SMAD2 and -3 in the induction of extracellular matrix proteins and proteases in response to TGFβ (Derynck and Zhang, 2003).
- SMAD proteins cooperate with the WNT pathway, both in a TGFβ-independent and -dependent manner. We already mentioned that in the absence of a TGFβ signal, SMAD4 interacts with LEF1/β-catenin and this complex activates expression of MYC (Figure 17-18, for details of the WNT pathway return to Figure 12-1, Chapter 12, “WNT Signaling and the Regulation of Cell Adhesion and Differentiation”) (Lim and Hoffmann, 2006). Cooperation between Smad and WNT in the presence of TGFβ occurs in expression of the *Xenopus* homeobox gene twin (Xtwn). Here Smad3 and -4 team up with tcf7l1/β-catenin in the transformation of dorsal mesoderm to form the Spemann organizer (Nishita et al., 2000).
- Inflammatory mediators, such as TNF, IFNγ, and IL1β, activate the transcription factors NFKB/RELA and IRF3, which combine with SMAD2 and -4, leading to the induction of the inhibitory SMAD7. In this way, they abrogate the response to BMP or TGFβ (see below and see Figure 17-14).
- Co-activators such as CREBBP or EP300 (Ogryzko et al., 1996) also serve as platforms for pathway cross-talk. An example is the collaboration between BMP2 and LIF, leading to activation of SMAD1/SMAD4 and STAT3, respectively, each binding to different sites in EP300 and cooperatively activating the glial fibrillary acidic protein promoter (Nakashima et al., 1999).

ID1 is required for progression through G1, one of its functions is to disable the action of Rb. ID proteins are helix–loop–helix proteins that lack a DNA-binding domain. They bind to other helix–loop–helix proteins and in so doing, prevent their access to DNA. In this way they prevent the action of, for instance, MyoD. Their expression is blocked in senescent fibroblasts.

Master transcription factors pave the way

Few TGFβ target genes are common to all cell types, such exceptions being the negative feedback regulators SMAD7 (inhibitor SMAD) and SKIL (SKI-like oncogene). Besides interference of other pathways that operate in any given cell, an essential determinant in the response to TGFβ is the epigenetic landscape of the cell concerned. These include DNA methylation marks, histone modifications, nucleosome positioning, and the presence of certain noncoding RNA copies. Together they shape the chromatin and dictate which enhancer/coding regions are open for transcription or not. A genome-wide mapping of SMAD3 binding to DNA in embryonic stem cell (ESC), myotubes, and pro-B cells revealed that a small set of master transcription factors direct SMAD3 to lineage-specific binding sites (Figure 17-12(b)). Master transcription factors, such as POU5F1 (formerly OCT4), MYOD1, and SPI1 (formerly PU.1) are expressed in large numbers (more than 30,000 copies per cell compared to 10,000 or less for "ordinary" transcription factors) and play an essential role in determining cell identity. They help direct SMAD3 binding by establishing accessible chromatin regions accommodating SMAD-binding elements and by physically attaching to them. For instance, for POU5F1 it was shown that the AGAC-nucleotide sequence recognized by SMAD proteins was enriched at POU5F1-binding sites (TTGCAT sequence) (Mullen et al., 2011; Figure 17-12(b)). Access of SMAD proteins to enhancer regions occupied by master transcription factors may be facilitated by the aforementioned action of the TRIM33/SMAD complex. Master transcription factors gained tremendous interest when it was shown that their ectopic expression could reprogram differentiated body cells into pluripotent stem cells (so-called induced stem cells) (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Theunissen and Jaenisch, 2014). Other examples of master transcription factors are NANOG, GATA4, HNF4a, or BRN2.

Embryonic stem cells (ESC) are obtained from the inner cell mass of mouse embryos at the blastocyst stage. They are pluripotent stem cells.

Myotubes are a syncytium of numerous myoblasts. They are the precursors of muscular fibers.

Pro-B cells are cells in the earliest stage of B cell development in the bone marrow. They are characterized by incomplete immunoglobulin heavy-chain arrangements and are defined as AA4$^+$, CD19$^+$, CD43$^+$, and cytoplasmic immunoglobulin M (IgM)$^-$.

HOLDING THE TGFβ PATHWAY IN CHECK

Dissociating the SMAD complex by ubiquitinylation of SMAD4

Although the residence time of SMAD binding to chromatin has not been measured, it is estimated that transcription factor residence times on chromatin are in the range of 30–120 s (Hager et al., 2009). The detached transcription factors may return to DNA, they may be removed from the nucleus or be destroyed. Over time this leads to transcription waves. Destabilization of the SMAD complex is facilitated by ubiquitinylation of SMAD4 at lysines K507 and K519 (human). This occurs through TRIM33, a chromatin-reader that we have already discussed above. It is generally assumed that the increase in histone acetylation, in particular at residues K9, 14, 18, 23, and 27 (as a consequence of the SMAD-mediated recruitment of EP300 and CREBBP), attracts an increasing number of TRIM33 to nucleosomes. The protein has E3-ubiquitin protein ligase activity (Dupont et al., 2005) and, together with an E2-ubiquitin-conjugating enzyme (for instance UBE2D3), causes mono-ubiquitinylation of SMAD4 in the MH2 domain. This leads to complex dissociation and detachment from DNA of the entire complex (Agricola et al., 2011). SMAD4 can be re-empowered through de-ubiquitinylation by USP9X (Dupont et al., 2009). However, as we will show below, SMAD2 and -3 are also modified and as a consequence they may no longer be available for complex formation.

Dephosphorylation of SMAD2 and -3

A steady-state level of phosphorylation of SMAD2 and -3 is achieved in cells within 15–30 min of adding TGFβ and this can last for several hours. Addition of a receptor kinase inhibitor, to interrupt the TGFR1 signal, then allows rapid (within 30 min) dephosphorylation (Inman et al., 2002). The SMAD-specific serine/threonine phosphatase PPM1A, member of the PP2C subfamily, is present in both the cytosol and the nucleus (Das et al., 1996). Of many phosphatases tested, only PPM1A is reactive against SMAD2 and -3. It causes dephosphorylation of the C-terminal SxS motif, preventing re-association of the SMAD proteins and allowing export of the individual components from the nucleus (Figure 17-13).

PPM1A affects transcription and other responses. For instance, in keratinocytes depleted of PPM1A, repression of MYC is increased and expression of the cell cycle inhibitors—CDKN2B (formerly p15$^{\mathrm{INK4b}}$) and CDKN1A (formerly p21$^{\mathrm{CIP1}}$)—in response to TGFβ is enhanced (Lin et al., 2006). It is likely that PPM1A is constitutively active so that the level of nuclear SMAD is determined by receptor activity. The picture that emerges for SMAD signaling is thus similar to that described for the STAT signaling pathway, in which the transcription factors act as remote sensors of receptor activity

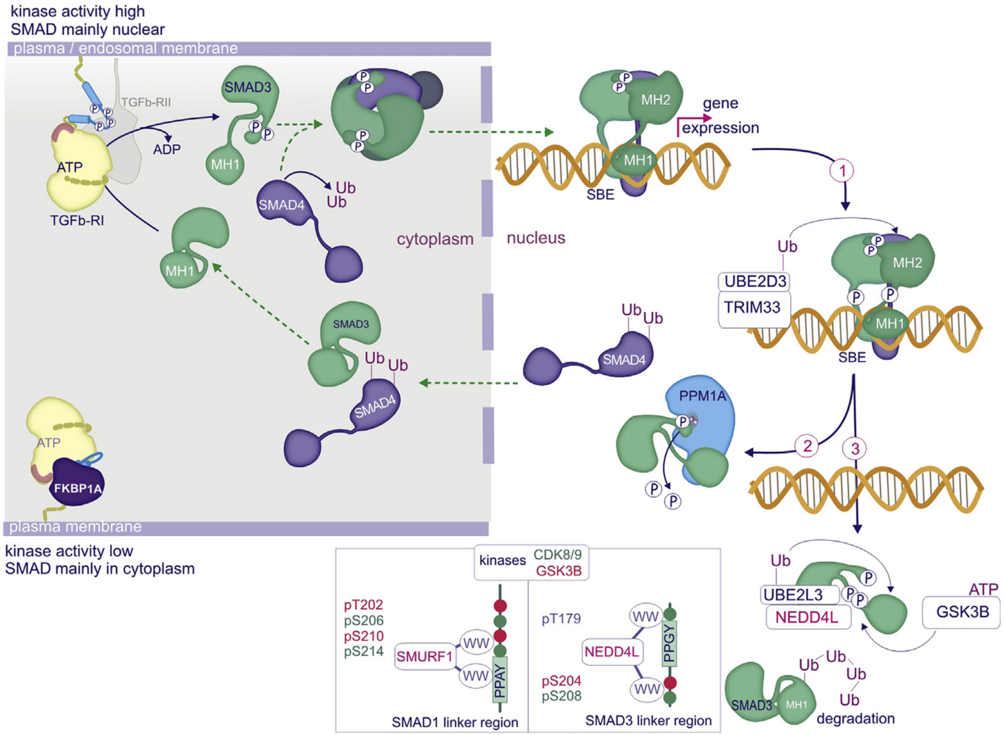

FIGURE 17-13 Disabling and re-enabling the SMAD transcription complex. Nuclear SMAD transcription complexes are rendered instable by mono-ubiquitinylation of SMAD4 in the MH2 domain. The reaction is catalyzed by TRIM33 (E3-ubiquitin ligase) in collaboration with an E2-ubiquitin-conjugating enzyme (for instance UBE2D3). The SMAD complex dissociated and becomes vulnerable to the action of the serine/threonine phosphatase PPM1A. Once dephosphorylated, the SMAD proteins transfer as monomers to the cytoplasm. Under conditions of low receptor activity, they remain here for some time but with high receptor activity, they are immediately re-phosphorylated and return to the nucleus as hetero-oligomeric transcription factor complexes. However, the regulated SMAD proteins, example shown of SMAD3, can also be phosphorylated by GSK3, in a follow-up of CDK8/9 phosphorylation, and this creates a binding site for E3-ubiquitin ligases. One of the two WW domains of NEDD4L recognizes the phosphothreonine-179 and the other the phosphoserine-204 and -208. NEDD4L teams up with the E2-ubiquitin-conjugation enzyme and this leads to the assembly of a K48-type poly-ubiquitin chain that marks the protein for destruction. The WW domains of SMURF1 recognize PPAY and phospo-serine-210 and -214 in the linker-region of MAD1 and the consequence is the same.

(return to Chapter 15, "Activating the Adaptive Immune System," Section "Down-regulation of the Jak–Stat Pathway," Figure 15-10).

We also note that PDP (pyruvate dehydrogenase phosphatase, essentially a mitochondrial enzyme but also present in the nucleus) is reactive against the SxS motif of SMAD1 (BMP pathway) but not of SMAD2 or -3 (TGFβ/activin pathway) (Chen et al., 2006). Finally, as we have already mentioned previously, the linker region of SMAD1 and SMAD3 are dephosphorylated by protein phosphatases that associate with the RNA-polymerase II, CTDSP1, CTDSP2, and CTDSPL, but they may also dephosphorylate the C-terminal SxS motif of SMAD1 (Sapkota et al., 2006).

Ubiquitinylation of R-SMAD proteins after phosphorylation of the linker region by GSK3B

Phosphorylation of the linker region by CDK8 and -9 causes the binding of Lin1 or YAP1, return to Figure 17-10, but it also primes for further phosphorylation by the protein kinase GSK3B. This protein kinase requires a phosphoserine positioned four residues downstream of the target residue (return to Chapter 16, "Signaling through the Insulin Receptor," Figure 16-11). GSK3B-mediated phosphorylation creates new binding sites for WW domains, this time for the E3-ubiquitin ligase SMURF1, which recognizes the modifications in the linker region of SMAD1, and NEDD4L, which binds SMAD3 (details are shown in Figure 17-13) (Gao et al., 2009). The ensuing poly-ubiquitinylation (assembly of a K48-chain) marks SMAD2 and -3 for recognition by the proteasome and leads to their destruction.

Inhibitory SMAD proteins

Another mechanism that leads to the attenuation of the TGFβ signal concerns the expression of the so-called inhibitory SMAD proteins, SMAD6 and -7. These are inhibitory because they lack the C-terminal SxS phosphorylation motif and have MH1 domains that are only distantly related (Figure 17-8). In the absence of TGFβ, they are retained in the nucleus. Both their expression and then their translocation into the cytoplasm are induced by TGFβ, activin, and BMP. Here, they act as inhibitors (Figure 17-14) (Nakao et al., 1997; Afrakhte et al., 1998). In the cytoplasm, SMAD6 forms complexes with phosphorylated SMAD1 thereby preventing its interaction with SMAD4 and, in this way, inhibiting BMP signaling (Hata et al., 1998).

For SMAD7, the mechanism is much more elaborate. It is bound to SMURF2 and, for reasons not fully understood, is protected against ubiquitylation in the nucleus. On addition of TGFβ, the SMAD7/SMURF2 complex moves into the cytosol to associate with the membrane through the C2 domain in SMURF2. Here, SMAD7 binds to the TGFBR1 and ubiquitylation ensues. The complex is internalized in caveolin-enriched vesicles

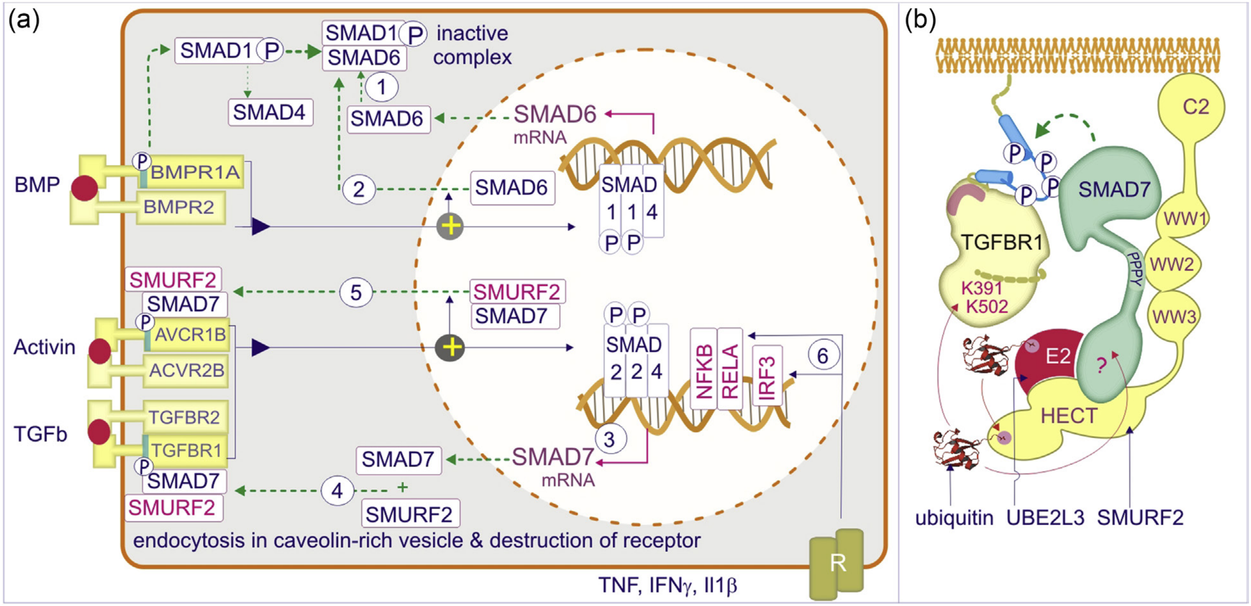

FIGURE 17-14 Induction of inhibitory Smad proteins. (a) Transcripts induced by members of the TGFβ-family include those coding for SMAD6 and -7. The newly expressed SMAD6 associates with phospho-SMAD1, preventing the formation of functional SMAD1/SMAD4 complexes (1). BMP also causes the nuclear export of existing SMAD6 (2) leading to the formation of the same nonfunctional complexes. The newly synthesized SMAD7 protein associates with SMURF2 and then with the type-I receptor. It causes degradation of the SMAD/receptor complex (steps 3 and 4). TGFβ or activin also causes nuclear export of existing Smad7/Smurf complexes (5). Expression of SMAD7 is also induced by inflammatory mediators such as TNF, IFNγ, or IL1β and these may act to attenuate the TGFβ response (6). (b) SMAD7 is an essential component of the SMURF2 ubiquitinylation complex. It brings together SMURF2, an E3-ubiquitine ligase, and the E2-conjugating protein UBE2L3. From here ubiquitin is transferred to the HECT domain of SMURF2 and then onto TGFBR1. SMURF7 acts as the receptor component of the complex, seeking out the substrate. Other proteins interacting with SMAD7 are potential targets. E2 represents UBE2L3, the ubiquitin donor associated with SMARF2.

and enters a destruction path through proteasomal degradation and perhaps also through fusion of the endosome with primary lysosomes (Ogunjimi et al., 2005; Izzi and Attisano, 2004; Di Guglielmo et al., 2003; Figure 17-14). The TGFBR1-associated SMAD7 also recruits USP15, an ubiquitin-protease which opposes the action of SMURF2, thus holding in check the pathway that holds in check the TGFβ-pathway (Inui et al., 2011).

The expression of SMAD7 is also induced by inflammatory mediators (such as TNF, INFγ, or IL1β) via the intermediate of NFKB/RELA and IRF3 transcription factors. This may be one mechanism by which these mediators lift the generally immunosuppressive influence of TGFβ (Shull et al., 1992; Wahl et al., 2006). Return to Chapter 13, "Activation of the Innate Immune System," for more information about the NFKB pathway.

It seems that SMAD7 originated as a transcription factor and then evolved to become a structural component of the SMURF ubiquitinylation complex. Here it acts as a receptor, selecting the substrate, and also enables the association of the E2-ubiquitin-conjugation protein UBE2L3 (formerly UbcH7) (Figure 17-14(b)). In view of this, it is possible that other SMAD-interacting proteins are targets of ubiquitin-mediated destruction. The finding that SMAD7/SMURF2 also binds β-catenin, causing its degradation, provides evidence for yet another mechanism of pathway cross-talk (Han et al., 2006).

BAMBI, a signal inhibitory pseudo-receptor

BMP, activin, and TGFβ induce the expression of BAMBI. Its expression correlates inversely with the metastatic potential of human melanoma cell lines (Degen et al., 1996). It is a truncated type-I receptor (closely resembles BMPRI) that binds ligand but lacks GS- and a protein kinase domain. However, the cytosolic segment binds TGFBR1 receptor and in so doing prevents its phosphorylation by type-II receptors. The effect is reduced signaling output from TGFβ receptors (return to Figure 17-6(e)) (Onichtchouk et al., 1999). It thus acts as a pseudo-receptor.

Sequestration of ligand and opposition by FGF prevent BMP signaling and causes dorsalization of *Xenopus laevis* embryos

We end this section with an example from developmental biology in which the action of bone morphogenetic protein, BMP, is attenuated at two levels. One through the production of ligand traps, noggin and chordin, and the other through destruction of SMAD proteins before they even had the chance of entering the nucleus. This action is essential for dorsal structures (spine, brain) in the development of frogs (*Xenopus laevis*).

We already mentioned that the linker region of the receptor-regulated SMAD protein contains numerous phosphorylation sites which are targeted by various kinases, among which are CDK4, -8, -9, GSK3B, and also MAP kinases (MAPK1, MAPK8, and MAPK14). Phosphorylation of SMAD1 by MAP kinases causes cytosolic retention and subsequent degradation (Matsuura et al., 2004; Sapkota et al., 2007). Phosphorylation of SMAD1 by MAPK1 (also known as ERK2) has been studied in more detail and we treat this topic in the context of neural development in *Xenopus laevis* (Figure 17-15; Kretzschmar et al., 1997; Pera et al., 2003).

In the early gastrula stage, the dorsal ectoderm transforms into neuroectoderm, which, at a later point provides the neural plate from which the notochord develops (De Robertis et al., 2000). Mainly, the ectoderm develops into dermis under the influence of bmp (gene names are written in lower case in *Xenopus*) but at the dorsal site of the embryo, the signal is opposed by fgf8, migrating from the mesodermal marginal zone (Streit et al., 2000; Delaune et al., 2005) and by chordin and noggin (and other factors) that trap the bmp ligand. These diffuse from the Spemann organizer at the onset of gastrulation (Figure 17-16). Fgf8 opposes the action of bmp and it also presents a neuro-inductive signal. The opposing action of fgf8 is mediated through its receptor tyrosine kinase (fgfr), activating the ras-mapk pathway and resulting in phosphorylation of the linker region of Smad1. This favors the interaction of Smad1 with Smurf1, an E3-ubiquitin ligase, thereby blocking the nuclear import-binding site located in the MH2 domain (no longer recognized by nup214). Smad1 fails to enter the nucleus and is degraded following ubiquitinylation (Sapkota et al., 2007). There are other instances in which the activities of the fgf8 and bmp pathways have opposing effects. These include the pairs fgf4/bmp2 in limb bud formation, fgf10/bmp4 in lung morphogenesis, fgf2/bmp4 in cranial suture fusion, and fgf8/bmp4 in the initiation of tooth development (Nakashima and Reddi, 2003). These processes may also be coordinated by signal integration at the level of Smad-1.

BMP: These proteins are involved in the development of the craniofacial complex (teeth, peridontium, and jaws). The discovery of stem cells in dental pulp now offers the possibility of regenerative therapy of the craniofacial complex. These rather fancy words disguise the idea that this approach may ultimately offer the possibility of regenerating whole teeth after extraction (Nakashima and Reddi, 2003).

In mammals, the linker region of SMAD3 is also phosphorylated by the cell-division cycle kinases CDK2 and CDK4. As their phosphorylation sites overlap with those of MAP kinases, the consequences are similar and SMAD3 remains sequestered in the cytoplasm to face destruction by SMURF1. Tumor cells that overexpress these kinases may therefore

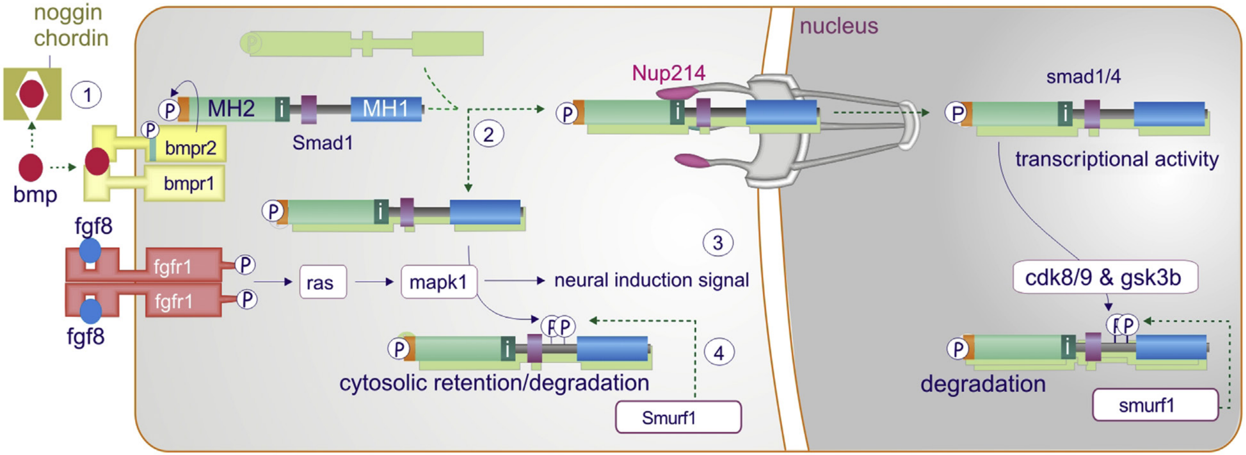

FIGURE 17-15 Opposing effects of BMP and FGF8 signaling on the translocation of Smad complexes into the nucleus. In embryonic development, the bmp signal is counteracted by secretion of trapping proteins such as noggin and chordin and also by fgf8 (1). This, through the Ras–MAPK pathway causes phosphorylation of serine residues in the linker region (2). Mapk1-mediated phosphorylation of Smad1 facilitates the binding of Smurf1 to PPxY (3) in a manner described in Figure 17-13. The Smurf/Smad complex cannot bind the nuclear pore protein Nup214 and remains in the cytoplasm. Also, Smurf1, an E3-ubiquitin ligase, causes destruction of Smad. In the nucleus, linker phosphorylation of Smads and Smurf-mediated destruction also occurs (4) and the kinases involved are cdk8/9 and gsk3b.

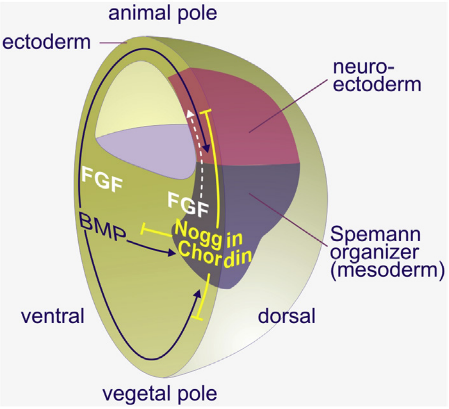

FIGURE 17-16 Neuro-ectoderm induction through fgf and the release of the bmp-traps noggin and chordin. Neuro-ectoderm formation in *Xenopus laevis* occurs through inhibition of the bmp signal, by release of noggin and chordin from the Spemann organizer, and through diffusion of fgf8, from the mesoderm marginal zone, into dorsal ectoderm. Neuro-ectoderm develops into notochord and gives rise to the brain.

be less sensitive to the growth inhibitory effect of TGFβ (Matsuura et al., 2004). For the record, the transcriptional activity of the SMAD proteins is also modulated by PRKCA (protein kinase C)-mediated phosphorylation of the DNA-binding MH1 domain (inhibition) and by binding of Ca^{2+}/calmodulin (enhancement) (Yakymovych et al., 2001). Ca^{2+}/calmodulin enhances the ventral mesoderm-forming activity of SMAD1 and blocks the activity of SMAD2. Moreover, calmodulin prevents MAPK1-mediated phosphorylation of SMAD1 and -2 and, vice versa, phosphorylation by MAPK1 inhibits the binding of calmodulin (Scherer and Graff, 2000).

Hans Spemann and the organizer

"How does that harmonious interlocking of separate processes come about which makes up the complete process of development? Do they go on side by side independently of each other (by 'self-differentiation,' Roux), but from the very beginning so in equilibrium that they form the highly complicated end product of the complete organism (the 17th century doctrine of preformation), or is their influence on each other one of mutual stimulation, advancement, or limitation?"

The question was put by Hans Spemann, professor of zoology at the University of Freiburg-im-Breisgau (-citation from the Nobel lecture of Spemann at http://nobelprize.org/nobel_prizes/medicine/laureates/1935/). With his graduate student, Hilde Mangold, he examined the transplantation of structures from the giant embryos of amphibians. Portions of surface cells (presumptive ectoderm) were removed and then implanted into an intact embryo. In many instances the implanted cells adopted the fate of the cells surrounding the new insertion site, but cells

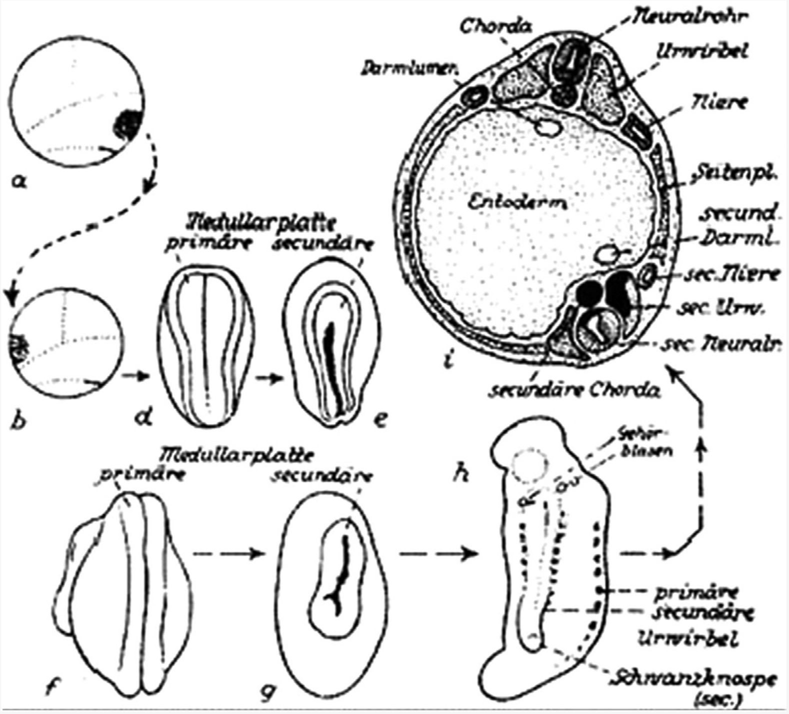

FIGURE 17-17 Secondary dorsal axis formation after implanting "the organizer."

from a limited area, namely the region of the upper and lateral blastopore lip, failed to do so. These gave rise to the formation of a nearly complete secondary dorsal axis, commencing with the formation of a second medullary plate (*Medullarplatte*) and giving rise to the formation of a secondary: neural tube (*Neuralrohr*), notochord (*chorda*), pre-vertebral discs (*Urwirbel*), kidneys (*Niere*), and even gut (*Darmlumen*). This area was named "organizer" and we now know it as the "Spemann organizer" (Figures 17-16 and 17-17)

Of several 100 chimeric embryos, only five survived, and these formed the basis for the famous paper that was published in 1924 (Spemann and Mangold, 1924). Spemann and Mangold were the first to prove the reality of "induction," by which one group of cells influences the developmental fate of the other. The identity of the inducing signal long remained the holy grail of developmental biology, but now, after about 80 years, we begin to realize that it constitutes a number of secreted factors, among which the antagonists of BMP play an important role.

In 1935, Spemann was awarded the Nobel Prize in Physiology or Medicine "for his discovery of the organizer effect in embryonic development." He was relieved of his professorial position in 1937, his place being taken by Otto Mangold, more acceptable to the regime and previously the husband of Hilde who had been killed in a kitchen explosion 13 years earlier.

TGFβ: TUMOR SUPPRESSOR AND METASTATIC PROMOTER?

Inhibition of cell proliferation

A common response to TGFβ in epithelial cells, inhibition of the cell division cycle, occurs by enhancing the expression of inhibitors of cyclin-dependent protein kinases. This is mediated through a self-enabling process in which SMAD proteins first repress the expression of MYC and then upregulate expression of the inhibitors. This has been studied in detail for CDKN1A (formerly $p21^{Cip1}$) and CDKN2B (formerly $p15^{INK4B}$) (Seoane et al., 2001, 2004). The MYC promoter contains two TCF/LEF-binding elements (TBE) that bind LEF1/CTNNB1 (β-catenin) and SMAD4 and drive gene expression. The MYC promoter region also contains a degenerate SMAD-binding sequence, TGFβ-inhibitory element (TIE) (Figure 17-18(a); Seoane et al., 2001; Frederick et al., 2004). In response to TGFβ, the inhibitory element binds SMAD3 and -4 combined with E2F4 (or E2F5). This complex then recruits the co-repressor RBL1 (formerly p107), all of which leads to effective silencing of MYC (Figure 17-18(b); Chen et al., 2002). Since the promoter region of the two cell cycle inhibitors contains an MYC/ZBTB17 (formerly Miz)-binding element that represses its transcription, loss of MYC abrogates this negative control and leaves the way open for SMAD-mediated activation of transcription. This occurs through two promoter elements, SBE-binding SMAD3 and -4 and FHBE which binds the forkhead transcription factor FOXO1 (Lim and Hoffmann, 2006). Together, these recruit EP300 which opens the way for the RNA polymerase. Elevated levels of CDKN1A ($p21^{CIP1}$) and CDKN2B ($p15^{INK4b}$) then inhibit the cell cycle through binding and inhibiting the kinase complex CCNE1 (cyclin E)/CDK2 that facilitates progression through the G1 phase.

Mutations of TGFβ signal components involved in cancer

Given the importance of TGFβ in suppressing cell proliferation, terminal differentiation of hematopoietic cells and the activation of cell death mechanisms, it is perhaps not surprising that mutations in the components

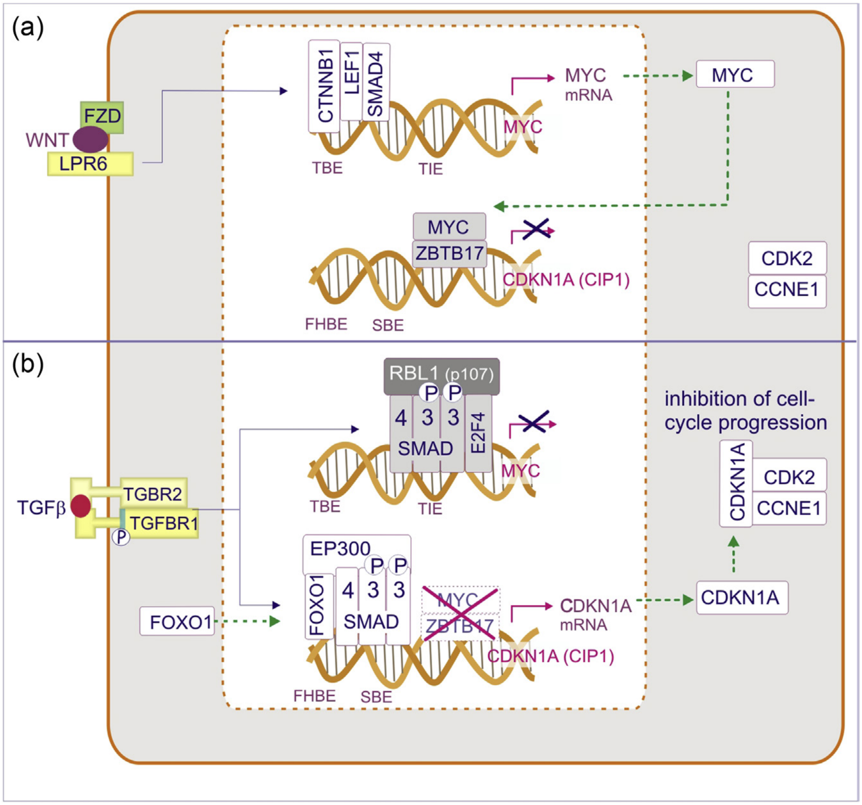

FIGURE 17–18 A self-enabling response regulates expression of the cell cycle inhibitor CKDN1A (p21CIP1). (a) Expression of the cyclin-dependent kinase inhibitor CDKN1A (p21^{CIP1}) is repressed by the transcription factors MYC and ZBTB17, both expressed through the influence of β-catenin/LEF1 complexed with SMAD4. (b) TGFβ inverses the situation. SMAD3/SMAD4 binds the TGFβ-inhibitory element in the MYC promoter. With E2F4 (or E2F5) and RBL1 they repress expression of MYC. The MYC/ZBTB17-mediated repression is lifted and a new transcriptional complex, comprising SMAD3/SMAD4, FOXO1, and EP300, drives expression of CDKN1A. After translation the inhibitor binds to CDK2/CCNE1 and puts a break on progression of the G1 phase of the cell division cycle. Regulation of another inhibitor, CDKN2B (p15^{INK4B}) is similar.

of the signal transduction pathway can increase susceptibility to oncogenic transformation. Together with other mutations that favor proliferation, these may ultimately result in the formation of tumors. Indeed, the common mediator SMAD4 was initially identified as one of the mutated or deleted genes linked to pancreatic, colorectal, or other carcinomas (Miyaki et al., 1999). Most of these mutations are present in the MH2 domain, which contains the SxS phosphorylation motif. They act to prevent complex formation with other SMAD proteins, decrease the stability of the protein, or prevent its interaction with non-SMAD transcriptional partners.

The importance of SMAD4 in colon cancer has been extensively studied in mice. Those that lack a single allele develop gastric/intestinal tumors within one year. These reveal a slow but continuous progression from initial hyperplastic lesions to more advanced stages with clear dysplasia, similar to human colorectal cancer. In more advanced stages, the cells also lose the second (functional) SMAD4 allele (Alberici et al., 2006). Mice lacking one allele of both APC (WNT pathway) and SMAD4 develop tumors earlier and they die earlier, suggesting that TGFβ-signaling is a component of APC-driven progression toward malignancy. There are also hereditary and somatic forms of colorectal cancer in which the TGFBR2 receptor is mutated, so that the cells lack their normal growth-inhibitory signaling mechanism.

Paradoxically, properly functional SMAD4 is also required for TGFβ-induced epithelial to mesenchymal transition, a process generally considered to be tumorigenic. In breast cancer, this transition is linked with metastasis (Deckers et al., 2006). Pancreatic tumor cells that are still sensitive to TGFβ-mediated growth inhibition become highly metastatic with overexpression of the TGFBR1 receptor (Schniewind et al., 2007). The general view is that TGFβ acts as a tumor suppressor by holding proliferation in check, thus reducing the chance of transforming mutations (fewer replication cycles, fewer errors). On the other hand, for cells already partly transformed (resistant to apoptosis and having reduced requirement of growth factors for proliferation), TGFβ may act as a metastasis promoter due to its capacity to induce the epithelial to mesenchymal transition (Oft et al., 2002).

Allowing cancer cells to escape immunosurveillance

High levels of TGFβ also enable transformed cells to escape immunosurveillance. There is much evidence for a role of the immune system in the elimination of cells in their early stages of transformation, and mice that lack essential components of the innate or adaptive immune system are prone to develop tumors (Zitvogel et al., 2006). Transformed cells are recognized by cytotoxic T-lymphocytes (CTLs) (and other immune cells) and by tumor-specific antibodies, leading to complement- or NK-cell-dependent lysis. Activated CTLs recognize antigens on the tumor cell membrane so that they merge to form an immune synapse in which the CTLs release perforin and granzymes (serine proteases) (Figure 17-19). Perforin triggers the rapid uptake of the granzymes into enormous endosomal vesicles. The granzymes then escape and induce apoptosis by cleaving nuclear proteins. The cytotoxic T cell also exposes FAS ligand (FASLG1), which, by binding to its receptor (FAS) on the target cells, causes the recruitment and activation of caspases that cleave numerous cellular proteins and induce apoptosis. Finally, cytotoxic T cells release IFNγ which has cytolytic effects on transformed cells.

On average, by making serial contacts with multiple targets, a cytotoxic immune cell can kill four tumor cells, mainly by induction of apoptosis. However, serial killing is associated with a loss of their own content of

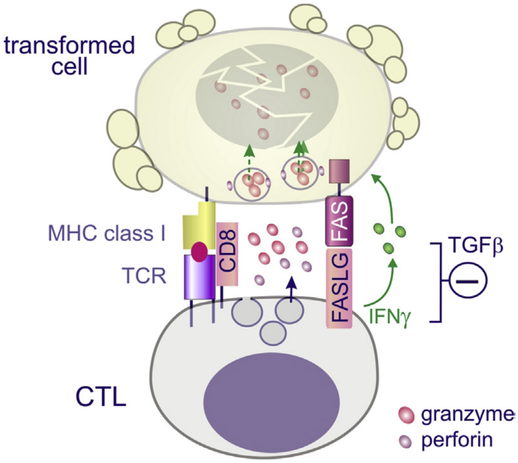

FIGURE 17–19 TGFβ suppresses immunosurveillance of transformed cells. Cytotoxic T-lymphocytes (CTLs) recognize transformed cell-antigens presented by MHC class I, through their T-cell receptor and CD8. Immune synapses are formed in which granules containing granzymes and perforin are released. Perforin facilitates the uptake of granzymes into large endocytic vesicles. Granzymes (serine proteases) escape and enter the nucleus, there to destroy proteins. The CTLs also present FASLG which binds the receptor FAS. Both the receptor engagement and the action of granzymes lead to cell death. Released interferon-γ (IFNγ) also has a cytolytic effect on transformed cells. TGFβ prevents expression of these cytolytic agents and therefore protects the transformed cell against killing.

perforin and granzymes, so the exhausted cells must replenish themselves in order to go on killing (Bhat and Watzl, 2007). This is where TGFβ protects tumor cells from being eliminated. It prevents replenishment of the granules by repressing transcription of the cytotoxic agents (Thomas and Massague, 2005). Alternatively, the effect is indirect. Neutralization of TGFβ with antibodies or soluble TGFBR2 receptors restores T-cell mediated tumor clearance.

TGFβ and the epithelial–mesenchymal transition: role of alternative pathways?

Epithelial–mesenchymal transition (EMT) is central to the morphogenic processes that occur in early embryonic development (Shook and Keller, 2003) and it also operates in the orchestration of metastasis in transformed cells. It involves the disaggregation of epithelial cell layers through dissolution of tight junctions, modulation of adherens junctions, reorganization of the actin cytoskeleton, and loss of cell polarity. TGFβ is one of the factors that drive the EMT. For more information about EMT, return to Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Cell Differentiation", Figure 12-02, Tables 12-01 and Section "Wnt targets with a Wnt-enhancer element."

SNAIL2 is Slug. Snail, so named because early *Drosophila* embryos that carry this lethal mutant resemble a snail. Slug, a vertebrate homolog of Snail, has roles in the formation of mesoderm during gastrulation and in the migration of neural crest cells. When the nomenclature was revised, snail became SNAIL1 and Slug became SNAIL2. For more on slugs and snails, see Barrallo-Gimeno and Nieto (2005).

Among the many genes induced by TGFβ1 are SNAIL2 and FZD1 (frizzled) (Zavadil et al., 2001). SNAIL2 is a transcription factor (Nieto et al., 1994) that represses expression of E-Cadherin (CDH1) leading to the loss of adherens junctions. FZD1 is a receptor for WNT that re-enforces the TGFβ signal by amplifying the gene responses that determine the mesenchymal phenotype (Luo et al., 2007). Other genes, in particular, those involved in matrix remodeling and cell motility are also induced by TGFβ1, but their expression requires the MAP-kinase pathway and they are thus indirect targets. Among these are metalloproteases (MMP1, MMP12), matrix components such as fibronectin, laminin and thrombospondin, and the integrin adhesion molecules. How the two pathways are connected has not been fully resolved.

In the process of EMT, TGFβ provokes the loss of tight junctions without involvement of SMAD proteins. The TGFBR1 receptor, retained at tight-junctions by occludin, binds to PARD6A, a scaffold protein that operates in the assembly of components that determine polarity (Figure 17-20; Ozdamar et al., 2005). TGFBR2 receptors are redistributed to the tight junctions on addition of TGFβ1, causing phosphorylation and activation of the TGFBR1 receptors and phosphorylation of PARD6A which now recruits the E3-ubiquitin ligase SMURF1. This leads to destruction of the monomeric GTPase RHOA and consequent dissolution of the tight junctions. The tissue dissociates, transforming into a mass of motile fibroblast-like cells. Loss of tight junctions also enables the expression of cyclin D, and by returning CDK4 to the nucleus, sets up a program that initiates the cell cycle.

NONCANONICAL PATHWAYS

We conclude this chapter by pointing out that the SMAD proteins do not possess the exclusive rights to the relaying of TGFβ signals into the cell. In addition to the PARD6/RHOA pathway already mentioned (Figure 17-20), type-II BMP receptors can directly activate LIMK1 which subsequently phosphorylates and inactivates the actin-binding/depolymerizing

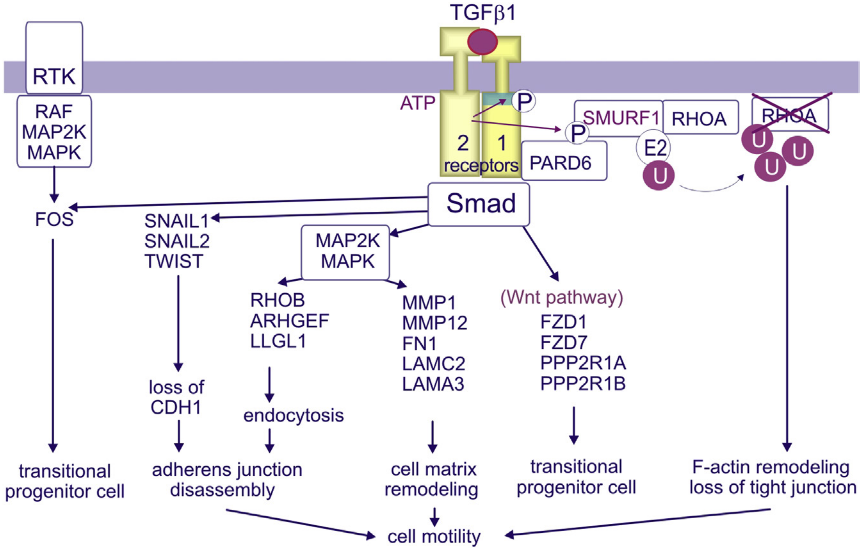

FIGURE 17–20 Genetic program underlying TGFβ-mediated epithelial–mesenchymal transition. In the process of epidermal to mesenchymal transition, there are 728 genes regulated by TGFβ. They can be grouped on the basis of the different signaling pathways which operate them and their different phenotypic outcomes. Numerous genes require a contribution of the MAP-kinase pathway. A selection of genes, those that have been discussed in this book, are presented. *Figure adapted from Ref. Zavadil et al. (2001).*

factors cofilin-1/CFL1, cofilin-2/CFL2, and destrin/DSTN. This pathway prevents cleavage of filamentous actin (F-actin) and therefore renders the actin-cytoskeleton less dynamic. The activity of LIMK plays a role in cell motility, cell cycle progression, and differentiation. The LIMK pathway may be enhanced in cells that lack components of the SMAD-pathway and it may contribute to the oncogenic transformation seen in colon epithelial cells in which SMAD4 is lost (Voorneveld et al., 2014). Independent of its kinase activity, TGFBR1 (sequence VPNEEDP) also recruits TRAF6 an E3-ubiquitin ligase which is associated with the E2-ubiquitin-conjugating enzyme complex UBE2N–UBE2V1. Receptor dimerization and perhaps oligomerization causes TRAF6-oligomerization and activation (we ignore how this works). This leads to K63-ubiquitinylation of TRAF6 itself and possible of the TGFBR1 receptor (two ubiquitinylation sites have been revealed, K391 and K502). The ensuing K63-ubiquitin chains are recognized by the ubiquitin-binding protein TAB1 which is associated with MAP3K7 (formerly TAK1, for TGFβ-activated kinase-1). These events initiate two MAPkinase pathways, terminating at JNK1 (MAPK8) and p38α (MAPK14) (the sequence is schematically represented in Figure 17-3) (Derynck and Zhang, 2003; Sorrentino et al., 2008). For full details, return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signaling through Ubiquitinylation," Sections "From TRAF6 to activation of NKFB" and "From TRAF to activation of MAPK8 (JNK1) and MAPK14 (p38α)." The TRAF6 pathway plays a critical nonredundant role in craniofacial development, and in embryonic neural crest-derived craniofacial cells, MAP3K7 is required not only for SMAD-independent TGF-β superfamily signaling but also for maximal ligand-induced C-terminal and linker phosphorylation of regulated SMAD-proteins (the noncanonical pathway reinforcing the canonical pathway) (Yumoto et al., 2013). As a last example, inactivating mutations of TGFBR1 or -2 do not necessarily lead to reduced TGFβ-signaling. This paradox is explained by the finding that the above-described noncanonical pathway is induced in a kinase-independent manner. A case in point is the Loeys–Dietz syndrome and its features are enlargement of the aorta with enhanced chances of aneurisms (Marfan syndrome), widely spaced eyes, cleft palate, malformation of the spine in the neck, club foot, and poor mineralization of bones. Part of the pathology is explained not by enhanced activity of the SMAD pathway but by an enhanced activity of MAP3K7 (Iwata et al., 2012; Kim et al., 2011).

References

Afrakhte, M., Moren, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C.H., Heldin, N.E., ten Dijke, P., 1998. Induction of inhibitory Smad6 and Smad7 mRNA by TGFbeta family members. Biochem. Biophys. Res. Commun. 249, 505–511.

Agricola, E., Randall, R.A., Gaarenstroom, T., Dupont, S., Hill, C.S., 2011. Recruitment of TIF1γ to chromatin via its PHD finger-bromodomain activates its ubiquitin ligase and transcriptional repressor activities. Mol. Cell 43, 85–96.

Alarcón, C., Zaromytidou, A.I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., Barlas, A., Miller, A.N., Manova-Todorova, K., Macias, M.J., Sapkota, G., Pan, D., Massagué, J., 2009. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 139, 757–769.

Alberici, P., Jagmohan-Changur, S., De Pater, E., van, d., Smits, V.,R., Hohenstein, P., Fodde, R., 2006. Smad4 haploinsufficiency in mouse models for intestinal cancer. Oncogene 25, 1841–1851.

Aragón, E., Goerner, N., Zaromytidou, A.-I., et al., 2011. A Smad action turnover switch operated by WW domain readers of a phosphoserine code. Genes Dev. 25, 1275–1288.

Aragon, E., Goerner, N., Xi, Q., Gomes, T., Gao, S., Massague, J., Macias, M.J., 2012. Stuctural basis for the versatile interactions of Smad7 with regulator WW domains in TGF-β pathways. Structure 20, 1726–1736.

Barrallo-Gimeno, A., Nieto, M.A., 2005. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132, 3151–3161.

Bassing, C.H., Yingling, J.M., Howe, D.J., Wang, T., He, W.W., Gustafson, M.L., Shah, P., Donahoe, P.K., Wang, X.F., 1994. Transforming growth factor beta type I receptor that signals to activate gene expression. Science 263, 87–89.

Bhat, R., Watzl, C., 2007. Serial killing of tumor cells by human natural killer cells - enhancement by therapeutic antibodies. PLoS One 2, e326.

Bork, P., Sudol, M., 1994. The WW domain: a signalling site in dystrophin? Trends Biochem. Sci. 19, 531–533.

Brown, C.B., Boyer, A.S., Runyan, R.B., Barnett, J.V., 1999. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. Science 283, 2080–2082.

de Caestecker, M.P., Yahata, T., Wang, D., Parks, W.T., Huang, S., Hill, C.S., Shioda, T., Roberts, A.B., Lechleider, R.J., 2000. The Smad4 activation domain (SAD) is a proline rich, P300-dependent transcriptional activation domain. J. Biol. Chem. 275, 2115–2122.

Chacko, B.M., Qin, B.Y., Tiwari, A., Shi, G., Lam, S., Hayward, L.J., De, C.M., Lin, K., 2004. Structural basis of heteromeric smad protein assembly in TGF-beta signaling. Mol. Cell 15, 813–823.

Chai, J., Wu, J.-W., Yan, N., Massague, J., Pavletich, N.P., Shi, Y., 2003. Features of a smad3 MH1-DNA complex. Roles of water and zinc in DNA binding. J. Biol. Chem. 278, 20327–20331.

Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeu, C., Massagué, J., Letarte, M., 1992. Endoglin is a component of the transforming growth factor–beta receptor system in human endothelial cells. J. Biol. Chem. 267, 19027–19030.

Chen, Y.G., Hata, A., Lo, R.S., Wotton, D., Shi, Y., Pavletich, N., Massagué, J., 1998. Determinants of specificity in TGF-β signal transduction. Genes Dev. 12, 2144–2152.

Chen, C.R., Kang, Y., Siegel, P.M., Massague, J., 2002. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. Cell 110, 19–32.

Chen, H.B., Shen, J., Ip, Y.T., Xu, L., 2006. Identification of phosphatases for Smad in the BMP/DPP pathway. Genes Dev. 20, 648–653.

Chen, W., Lam, S.S., Srinath, H., Schiffer, C.A., Royer, W.E., Lin, K., 2007. Competition between Ski and CREB-binding protein for binding to Smad proteins in transforming growth factor-β signaling. J. Biol. Chem. 282, 11365–11376.

Cheng, B., Price, D.H., 2007. Properties of RNA polymerase II elongation complexes before and after the P-TEFβ-mediated transition into productive elongation. J. Biol. Chem. 282, 21901–21912.

Das, A.K., Helps, N.R., Cohen, P.T., Barford, D., 1996. Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 A resolution. EMBO J. 15, 6798–6809.

De Robertis, E.M., Larrain, J., Oelgeschlager, M., Wessely, O., 2000. The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat. Rev. Genet. 1, 171–181.

Deckers, M., van Dinther, M., Buijs, J., Que, I., Lowik, C., van der, P.G., ten, D.P., 2006. The tumor suppressor Smad4 is required for transforming growth factor β-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res. 66, 2202–2209.

Degen, W.G., Weterman, M.A., van Groningen, J.J., Cornelissen, I.M., Lemmers, J.P., Agterbos, M.A., Geurts van, K.A., Swart, G.W., Bloemers, H.P., 1996. Expression of nma, a novel gene, inversely correlates with the metastatic potential of human melanoma cell lines and xenografts. Int. J. Cancer 65, 460–465.

Delaune, E., Lemaire, P., Kodjabachian, L., 2005. Neural induction in *Xenopus* requires early FGF signalling in addition to BMP inhibition. Development 132, 299–310.

Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425, 577–584.

Di Guglielmo, G.M., Le, R.C., Goodfellow, A.F., 2003. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat. Cell Biol. 5, 410–421.

ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., Heldin, C.H., 1994. Characterization of type I receptors for transforming growth factor-β and activin. Science 264, 101–104.

Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M., Piccolo, S., 2005. Germ-layer specification and control of cell growth by ectodermin, a Smad4 ubiquitin ligase. Cell 121, 87–99.

Dupont, S., Mamidi, A., Cordenonsi, M., Montagner, M., Zacchigna, L., Adorno, M., Martello, G., Stinchfield, M.J., Soligo, S., Morsut, L., et al., 2009. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitinylation. Cell 136, 123–135.

Durocher, D., Taylor, I.A., Sarbassova, D., Haire, L.F., Westcott, S.L., Jackson, S.P., Smerdon, S.J., Yaffe, M.B., 2000. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell 6, 1169–1182.

Estevez, M., Attisano, L., Wrana, J.L., Albert, P.S., Massagué, J., Riddle, D.L., Padgett, R.W., 1993. The daf-4 gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. Nature 365, 644–649.

Feng, X.H., Derynck, R., 1997. A kinase subdomain of transforming growth factor-beta (TGF-beta) type I receptor determines the TGF-beta intracellular signaling specificity. EMBO J. 16, 3912–3923.

Frederick, J.P., Liberati, N.T., Waddell, D.S., Shi, Y., Wang, X.F., 2004. Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol. Cell Biol. 24, 2546–2559.

Gao, S., Alarcon, C., Sapkota, G., Rahman, S., Chen, P.Y., Goerner, N., Macias, M.J., Erdjument–Bromage, H., Tempst, P., Massague, J., 2009. Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. Mol. Cell 36, 457–468.

Georgi, L.L., Albert, P.S., Riddle, D.L., 1990. Daf-1, a *C. elegans* gene controlling dauer larva development, encodes a novel receptor protein kinase. Cell 61, 635–645.

Gougos, A., Letarte, M., 1988. Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J. Immunol. 141, 1925–1933.

Goumans, M.J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S., ten, D.P., 2003. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol. Cell 12, 817–828.

Gray, P.C., Shani, G., Aung, K., Kelber, J., Vale, W., 2006. Cripto binds transforming growth factor beta (TGF-beta) and inhibits TGF-beta signaling. Mol. Cell Biol. 26, 9268–9278.

Groppe, J., Hinck, C.S., Samavarchi-Tehrani, P., Zubieta, C., Schuermann, J.P., Taylor, A.B., Schwarz, P.M., Wrana, J.L., Hinck, A.P., 2008. Cooperative assembly of raTGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol. Cell 29, 157–168.

Hager, G.L., McNally, J.G., Misteli, T., 2009. Transcription dynamics. Mol. Cell 35, 741–753.

Han, G., Li, A.G., Liang, Y.Y., Owens, P., He, W., Lu, S., Yoshimatsu, Y., Wang, D., ten, D.P., Lin, X., Wang, X.J., 2006. Smad7-induced beta-catenin degradation alters epidermal appendage development. Dev. Cell 11, 301–312.

Hata, A., Lagna, G., Massague, J., Hemmati-Brivanlou, A., 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 12, 186–197.

He, W., Dorn, D.C., Erdjument-Bromage, H., Tempst, P., Moore, M.A., Massague, J., 2006. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell 125, 929–941.

Heldin, C.H., Miyazono, K., ten-Dijke, P., 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390, 465–471.

Hong, W., Guan, K.L., 2012. The YAP and TAZ transcription co-activators: key downstream effectors of the mammalian Hippo pathway. Semin. Cell Dev. Biol. 23, 785–793.

Huse, M., Muir, T.W., Xu, L., Chen, Y.G., Kuriyan, J., Massague, J., 2001. The TGFbeta receptor activation process: an inhibitor- to substrate-binding switch. Mol. Cell 8, 671–682.

Inman, G.J., Nicolas, F.J., Hill, C.S., 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-β receptor activity. Mol. Cell 10, 283–294.

Inui, M., Manfrin, A., Mamidi, A., Martello, G., Morsut, L., Soligo, S., Enzo, E., Moro, S., Polo, S., Dupont, S., Cordenonsi, M., Piccolo, S., 2011. USP15 is a deubiquitylating enzyme for receptor-activated SMADs. Nat. Cell Biol. 13, 1368–1375.

Iwafuchi-Doi, M., Zaret, K.S., 2014. Pioneer transcription factors in cell reprogramming. Genes Dev. 28, 2679–2692.

Iwata, J., Hacia, J.G., Suzuki, A., Sanchez-Lara, P.A., Urata, M., Chai, Y., 2012. Modulation of noncanonical TGF-β signaling prevents cleft palate in Tgfbr2 mutant mice. J. Clin. Invest. 122, 873–885.

Izzi, L., Attisano, L., 2004. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene 23, 2071–2078.

Janknecht, R., Wells, N.J., Hunter, T., 1998. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 12, 2114–2119.

Jones, P.A., Takai, D., 2001. The role of DNA methylation in mammalian epigenetics. Science 293, 1068–1107.

Kang, Y., Chen, C.R., Massague, J., 2003. A self-enabling TGFbeta response coupled to stress signaling: smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol. Cell 11, 915–926.

Kapoor-Vazirani, P., Kagey, J.D., Vertino, P.M., 2011. SUV420H2-mediated H4K20 trimethylation enforces RNA polymerase II promoter-proximal pausing by blockinghMOF-dependent H4K16 acetylation. Mol. Cell Biol. 31, 1594–1609.

Kato, Y., Habas, R., Katsuyama, Y., Naar, A.M., He, X., 2002. A component of the ARC/mediator complex required for TGFbeta/Nodal signalling. Nature 418, 641–646.

Kim, D., Schoenhoff, F., Cohn, R.D., Loeys, B.L., Thomas, C.J., Patnaik, S., Marugan, J.J., Judge, D.P., Dietz, H.C., 2011. Noncanonical TGFβ signaling contributes to aortic aneurysm progression in Marfan syndrome mice. Science 332, 358–361.

Klingberg, F., Chow, M.L., Koehler, A., Boo, S., Buscemi, L., Quinn, T.M., Costell, M., Alman, B.A., Genot, E., Hinz, B., 2014. Prestress in the extracellular matrix sensitizes latent TGF-β1 for activation. J. Cell Biol. 207, 283–297. http://www.ncbi.nlm.nih.gov.gate2.inist.fr/pubmed/?term=Prestress+in+the+extracellular+matrix+sensitizes+latent+TGF-%CE%B21+for+activation.

Kretzschmar, M., Massague, J., 1998. SMADs: mediators and regulators of TGF-beta signaling. Curr. Opin. Genet. Dev. 8, 103–111.

Kretzschmar, M., Doody, J., Massague, J., 1997. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389, 618–622.

Lebrin, F., Goumans, M.J., Jonker, L., Carvalho, R.L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H.M., ten, D.P., 2004. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J. 23, 4018–4028.

Lee, T.I., Young, R.A., 2013. Transcriptional regulation and its misregulation in disease. Cell 152, 1237–1251.

Lewis, K.A., Gray, P.C., Blount, A.L., MacConell, L.A., Wiater, E., Bilezikjian, L.M., Vale, W., 2000. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404, 411–414.

Lim, S.K., Hoffmann, F.M., 2006. Smad4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor to increase c-myc expression in the absence of TGF-beta signaling. Proc. Natl. Acad. Sci. U.S.A. 103, 18580–18585.

Lin, H.Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R.A., Lodish, H.F., 1992. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. Cell 68, 775–785.

Lin, X., Duan, X., Liang, Y.Y., Su, Y., Wrighton, K.H., Long, J., Hu, M., Davis, C.M., Wang, J., Brunicardi, F.C., Shi, Y., Chen, Y.G., Meng, A., Feng, X.H., 2006. PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. Cell 125, 915–928.

Ling, N., Ying, S.Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., Guillemin, R., 1986. Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. Nature 321, 779–782.

Liu, F., Hata, A., Baker, J.C., Doody, J., Carcamo, J., Harland, R.M., Massagué, J., 1996. A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 381, 620–623.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S., Massague, J., 1991. Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell 67, 785–795.

Lopez-Casillas, F., Wrana, J.L., Massague, J., 1993. Betaglycan presents ligand to the TGF beta signaling receptor. Cell 73, 1435–1444.

Luo, K., Lodish, H.F., 1996. Signaling by chimeric erythropoietin-TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF- beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. EMBO J. 15, 4485–4496.

Luo, W., Peterson, A., Garcia, B.A., Coombs, G., Kofahl, B., Heinrich, R., Shabanowitz, J., Hunt, D.F., Yost, H.J., Virshup, D.M., 2007. Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex. EMBO J. 26, 1511–1521.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. Science 298, 1912–1934.

Massague, J., Seoane, J., Wotton, D., 2005. Smad transcription factors. Genes Dev. 19, 2783–2810.

Mathews, L.S., Vale, W.W., 1991. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65, 973–982.

Matsuura, I., Denissova, N.G., Wang, G., He, D., Long, J., Liu, F., 2004. Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature 430, 226–231.

McAllister, K.A., Grogg, K.M., Johnson, D.W., Gallione, C.J., Baldwin, M.A., Jackson, C.E., Helmbold, E.A., Markel, D.S., McKinnon, W.C., Murrell, J., 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat. Genet. 8, 345–351.

McPherron, A.C., Lee, S.J., 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. U.S.A. 94, 12457–12461.

Melhuish, T.A., Wotton, D., 2000. The interaction of the carboxyl terminus-binding protein with the Smad corepressor TGIF is disrupted by a holoprosencephaly mutation in TGIF. J. Biol. Chem. 275, 39762–39766.

Misra, S., Hurley, J.H., 1999. Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. Cell 97, 657–666.

Miyaki, M., Iijima, T., Konishi, M., Sakai, K., Ishii, A., Yasuno, M., Hishima, T., Koike, M., Shitara, N., Iwama, T., Utsunomiya, J., Kuroki, T., Mori, T., 1999. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. Oncogene 18, 3098–3103.

Moustakas, A., Heldin, C.-H., 2009. The regulation of TGFβ signal transduction. Development 136, 3699–3714.

Mullen, A.C., Orlando, D.A., Newman, J.J., Loven, J., Lumar, R.M., Bilodeau, S., Teddy, J., Guenther, M.G., DeKoter, R.P., Young, R.A., 2011. Master transcription factors determine cell-type-specific responses to TGF-β signaling. Cell 147, 565–576.

Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.E., Heldin, C.H., ten-Dijke, P., 1997. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389, 631–635.

Nakashima, N., Reddi, A.H., 2003. The application of bone morphogenetic proteins to dental tissue engineering. Nat. Biotech. 21, 1025–1032.

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., Taga, T., 1999. Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science 284, 479–482.

Newfeld, S.J., Chartoff, E.H., Graff, J.M., Melton, D.A., Gelbart, W.M., 1996. Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells. Development 122, 2099–2108.

Nieto, M.A., Sargent, M.G., Wilkinson, D.G., Cooke, J., 1994. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science 264, 835–839.

Nishita, M., Hashimoto, M.K., Ogata, S., Laurent, M.N., Ueno, N., Shibuya, H., Cho, K.W., 2000. Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. Nature 403, 781–785.

Oft, M., Akhurst, R.J., Balmain, A., 2002. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat. Cell Biol. 4, 487–494.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., Nakatani, Y., 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87, 953–959.

Ogunjimi, A.A., Briant, D.J., Pece-Barbara, N., 2005. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol. Cell 19, 297–308.

Oklu, R., Hesketh, R., 2000. The latent transforming growth factor beta binding protein (LTBP) family. Biochem. J. 352 (Pt. 3), 601–610.

Onichtchouk, D., Chen, Y.G., Dosch, R., Gawantka, V., Delius, H., Massague, J., Niehrs, C., 1999. Silencing of TGFbeta signalling by the pseudoreceptor BAMBI. Nature 401, 480–485.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y., Wrana, J.L., 2005. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science 307, 1603–1609.

Pera, E.M., Ikeda, A., Eivers, E., De Robertis, E.M., 2003. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev. 17, 3023–3028.

Pierreux, C.E., Nicolas, F.J., Hill, C.S., 2000. Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. Mol. Cell Biol. 20, 9041–9054.

Qin, B.Y., Chacko, B.M., Lam, S.S., de Caestecker, M.P., Correia, J.J., Lin, K., 2001. Structural basis of Smad1 activation by receptor kinase phosphorylation. Mol. Cell 8, 1303–1312.

Raftery, L.A., Twombly, V., Wharton, K., Gelbart, W.M., 1995. Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*. Genetics 139, 241–254.

Ramos, A., Camargo, F.D., 2012. The Hippo signaling pathway and stem cell biology. Trends Cell Biol. 22, 339–346.

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., De Larco, J.E., Todaro, G.J., 1980. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. U.S.A. 77, 3494–3498.

Ross, S., Cheung, E., Petrakis, T.G., Howell, M., Kraus, W.L., Hill, C.S., 2006. Smads orchestrate specific histone modifications and chromatin remodelling to activate transcription. EMBO J. 25, 4490–4502.

Sapkota, G., Knockaert, M., Alarcon, C., Montalvo, E., Brivanlou, A.H., Massague, J., 2006. Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. J. Biol. Chem. 281, 40412–40419.

Sapkota, G., Alarcon, C., Spagnoli, F.M., Brivanlou, A.H., Massague, J., 2007. Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol. Cell 25, 441–454.

Savage, C., Das, P., Finelli, A.L., Townsend, S.R., Sun, C.Y., Baird, S.E., Padgett, R.W., 1996. *Caenorhabditis elegans* genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor b pathway components. Proc. Natl. Acad. Sci. U.S.A. 93, 790–794.

Scherer, A., Graff, J.M., 2000. Calmodulin differentially modulates Smad1 and Smad2 signaling. J. Biol. Chem. 275, 41430–41438.

Schmierer, B., Hill, C.S., 2005. Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor-dependent nuclear accumulation of Smads. Mol. Cell Biol. 25, 9845–9858.

Schniewind, B., Groth, S., Sebens, M.S., Sipos, B., Schafer, H., Kalthoff, H., Fandrich, F., Ungefroren, H., 2007. Dissecting the role of TGF-beta type I receptor/ALK5 in pancreatic ductal adenocarcinoma: smad activation is crucial for both the tumor suppressive and prometastatic function. Oncogene 26, 4850–4862.

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., Massague, J., 2001. TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat. Cell Biol. 3, 400–408.

Seoane, J., Le, H.V., Shen, L., Anderson, S.A., Massague, J., 2004. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 117, 211–223.

Shen, M.M., Schier, A.F., 2000. The EGF-CFC gene family in vertebrate development. Trends Genet. 16, 303–309.

Shi, Y., Massague, J., 2003. Mechanisms of TGFbeta signaling from cell membrane to the nucleus. Cell 113, 685–700.

Shi, Y., Wang, Y.F., Jayaraman, L., Yang, H., Massague, J., Pavletich, N.P., 1998. Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell 94, 585–594.

Shilatifard, A., Conaway, R.C., Conaway, J.W., 2003. The RNA polymerase II elongation complex. Ann. Rev. Biochem. 72, 693–715.

Shiou, S.R., Singh, A.B., Moorthy, K., Datta, P.K., Washington, M.K., Beauchamp, R.D., Dhawan, P., 2007. Smad4 regulates claudin-1 expression in a transforming growth factor-beta-independent manner in colon cancer cells. Cancer Res. 67, 1571–1579.

Shook, D., Keller, R., 2003. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech. Dev. 120, 1351–1383.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., 1992. Targeted disruption of the mouse transforming growth factor-beta-1 gene results in multifocal inflammatory disease. Nature 359, 693–699.

Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C.H., Landstrom, M., 2008. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat. Cell Biol. 10, 1199–1207.

Spemann, H., Mangold, H., 1924. Über induction von embryonalanlagen durch implantation artfremder organisatoren. Wilhelm Roux Arch. Entw. Mech. Org. 100, 599–638.

Streit, A., Berliner, A.J., Papanayotou, C., Sirulnik, A., Stern, C.D., 2000. Initiation of neural induction by FGF signalling before gastrulation. Nature 406, 74–78.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.

Theunissen, T.W., Jaenisch, R., 2014. Molecular control of induced pluripotency. Cell Stem Cell 14, 720–734.

Thomas, D.A., Massague, J., 2005. TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 8, 369–380.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., Wrana, J.L., 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell 95, 779–791.

Valcourt, U., Kowanetz, M., Niimi, H., Heldin, C.H., Moustakas, A., 2005. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol. Biol. Cell 16, 1987–2002.

Varelas, X., Sakuma, R., Samavarchi-Tehrani, P., Peerani, R., Rao, B.M., Dembowy, J., Yaffe, M.B., Zandstra, P.W., Wrana, J.L., 2008. TAZ controls SMad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat. Cell Biol. 10, 837–848.

Voorneveld, P.W., Kodach, L.L., Jacobs, J.R., Liv, N., Zonnevylle, A.C., Hoogenboom, J.P., Biermond, I., Verspaget, H.W., et al., 2014. Loss of SMAD4 alters BMP signaling to promote colorectal cancer cell metastasis via activation of Rho and ROCK. Gastroenterology 147, 196–208.

Wahl, S.M., Wen, J., Moutsopoulos, N., 2006. TGFbeta: a mobile purveyor of immune privilege. Immunol. Rev. 213, 213–227.

Wang, X.-F., Lin, H.Y., Ng, E.E., Downward, J., Lodish, H.F., Weinberg, R.A., 1991. Expression cloning and characterization of the TGF-beta type III receptor. Cell 67, 797–805.

Wernig, L., Meissnerr, A., Foreman, R., Brambrink, T., Ku, M., Hochedlingerr, J., Bernstein, B.E., Jaenisch, R., 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448, 318–324.

Wiater, E., Harrison, C.A., Lewis, K.A., Gray, P.C., Vale, W.W., 2006. Identification of distinct inhibin and transforming growth factor β-binding sites on betaglycan: functional separation of betaglycan co-receptor actions. J. Biol. Chem. 281, 17011–17022.

Wu, M.Y., Hill, C.S., 2009. Tgf-beta superfamily signaling in embryonic development and homeostasis. Dev. Cell 16, 329–343.

Wu, G., Chen, Y.G., Ozdamar, B., Gyuricza, C.A., Chong, P.A., Wrana, J.L., Massague, J., Shi, Y., 2000. Structural basis of Smad2 recognition by the Smad anchor for receptor activation. Science 287, 92–97.

Wu, J.W., Hu, M., Chai, J., Seoane, J., Huse, M., Li, C., Rigotti, D.J., Kyin, S., Muir, T.W., Fairman, R., Massague, J., Shi, Y., 2001. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. Mol. Cell 8, 1277–1289.

Xi, Q., Wang, Z., Zaromytidou, A.-I., Zhang, X.H.-F., Chow-Tsang, L.-F., Liu, J.X., Kim, H., Barlas, A., Manova-Todorova, K., Kaartinen, V., Studer, L., Mark, M., Patel, D.J., Massagué, J., 2011. A poised chromatin platform for TGF-β access to master regulators. Cell 147, 1511–1524.

Xu, L., Kang, Y., Col, S., Massague, J., 2002. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGF-beta signaling complexes in the cytoplasm and nucleus. Mol. Cell 10, 271–282.

Yakymovych, I., ten Dijke, P., Heldin, C.H., Souchelnytskyi, S., 2001. Regulation of Smad signaling by protein kinase C. FASEB J. 15, 553–555.

Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., Xiong, X., Munger, J.S., 2007. Absence of integrin-mediated TGFβ1 activation in vivo recapitulates the phenotype of TGFβ1-null mice. J. Cell Biol. 176, 787–793.

Yumoto, K., Thomas, P.S., Lane, J., Matsuzaki, K., Inagaki, M., nimoiya-Tsuji, J., Scott, G.J., Ray, M.K., Ishii, M., Maxson, R., Mishina, Y., Kaartinen, V., 2013. TGFbeta-activated kinase 1 (TAK1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells. J. Biol. Chem. 288, 13467–13480.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y.C., Massimi, A., Kneitz, S., Piek, E., Bottinger, E.P., 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. Proc. Natl. Acad. Sci. U.S.A. 98, 6686–6691.

Zitvogel, L., Tesniere, A., Kroemer, G., 2006. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat. Rev. Immunol. 6, 715–727.

CHAPTER

18

Protein Phosphatases

INTRODUCTION

Phosphorylation of proteins serves multiple roles in the regulation of cell function. But this is only half the story. If the transfer of phosphate groups to proteins is to serve as a precise and sensitive-signaling mechanism then necessarily it must operate against a low background. Dephosphorylation, therefore, is as important as phosphorylation and it follows that the phosphoprotein phosphatases are integral components of the signaling systems operated by the protein kinases (Sun and Tonks, 1994). In a number of cases dephosphorylation serves as a true reset button, bringing proteins back to their inactive state.

A good example of this is the role of the serine/threonine phosphatase PPP1CA (formerly PP1c) which dephosphorylates glycogen phosphorylase *a* (PYGL in liver and PYGM in muscle), thereby terminating the breakdown of glycogen. There are other enzymes (e.g., glycogen synthase (GYS1 in muscle and GYS2 in liver), GSK3B, SRC, and LCK) or transcription factors (e.g., JUN and NFAT) that are inactive in their phosphorylated state, only becoming active as a consequence of dephosphorylation (Figure 18-1). In the case of LCK, a tyrosine-protein kinase, activation of the enzyme requires dephosphorylation of a C-terminal tyrosine residue and then phosphorylation of another tyrosine residue, in the activation segment through an autophosphorylation mechanism (subject discussed in Chapter 15, “Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases,” Figures 15-12 and 15-13). To become fully active, the transcription factor JUN undergoes dephosphorylation of serine and threonine residues near the DNA-binding site and phosphorylation of serines near the N-terminus. NFAT too needs to be dephosphorylated in order to expose its nuclear localization signal.

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Web resource

Consult the HUGO gene-nomenclature database for an overview of the big family of protein tyrosine phosphatases. URL: www.genenames.org/genefamilies/PTP.

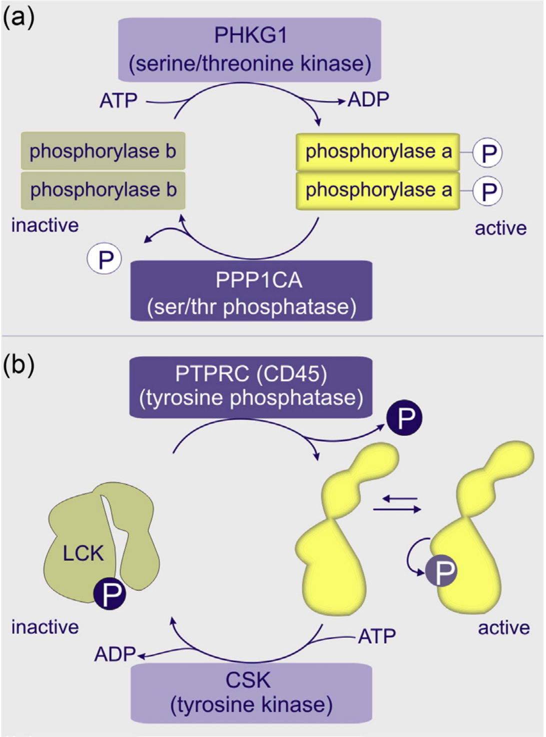
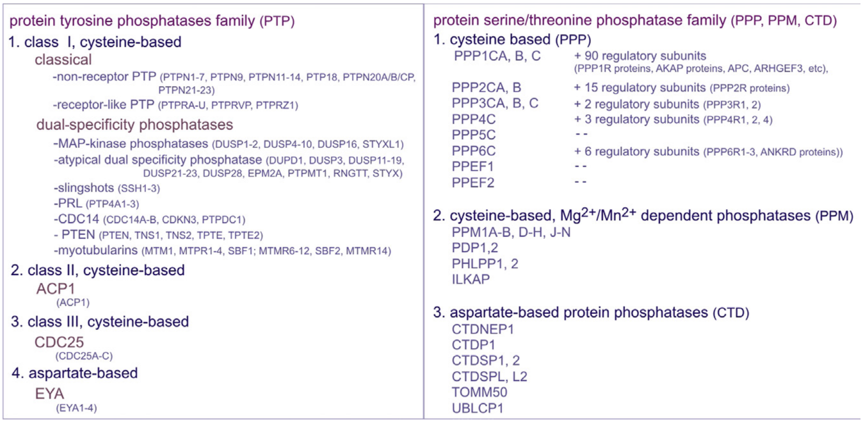


FIGURE 18-1 Phosphatases in the generation of signals for both deactivation and activation. (a) Protein phosphatases as a "reset button." The serine/threonine phosphatase PPP1CA dephosphorylates and deactivates phosphorylase A. (b) Protein phosphatase as activator. PTPRC (CD45), a receptor-like tyrosine phosphatase primes LCK for activation.

As for protein kinases, a first distinction is made between serine/threonine and tyrosine phosphates. Like protein kinases some phosphatases are dual specific, they recognize both phosphoserine/threonine and phosphotyrosine. In contrast to the protein kinases (both serine/threonine and tyrosine), which share a common ancestry, the tyrosine phosphatases and the serine/threonine-specific phosphatases bear little relation to each other. An overview of all human protein phosphatases is shown in Figure 18-2. In this chapter, we describe protein tyrosine



protein tyrosine phosphatases family (PTP)

1. class I, cysteine-based
    - classical
        - -non-receptor PTP (PTPN1-7, PTPN9, PTPN11-14, PTP18, PTPN20A/B/CP, PTPN21-23)
        - -receptor-like PTP (PTPRA-U, PTPRVP, PTPRZ1)
    - dual-specificity phosphatases
        - -MAP-kinase phosphatases (DUSP1-2, DUSP4-10, DUSP16, STYXL1)
        - -atypical dual specificity phosphatase (DUPD1, DUSP3, DUSP11-19, DUSP21-23, DUSP28, EPM2A, PTPMT1, RNGTT, STYX)
        - -slingshots (SSH1-3)
        - -PRL (PTP4A1-3)
        - -CDC14 (CDC14A-B, CDKN3, PTPDC1)
        - - PTEN (PTEN, TNS1, TNS2, TPTE, TPTE2)
        - -myotubularins (MTM1, MTPR1-4, SBF1; MTMR6-12, SBF2, MTMR14)
2. class II, cysteine-based
    - ACP1 (ACP1)
3. class III, cysteine-based
    - CDC25 (CDC25A-C)
4. aspartate-based
    - EYA (EYA1-4)

protein serine/threonine phosphatase family (PPP, PPM, CTD)

1. cysteine based (PPP)

PPP1CA, B, C	+ 90 regulatory subunits (PPP1R proteins, AKAP proteins, APC, ARHGEF3, etc),
PPP2CA, B	+ 15 regulatory subunits (PPP2R proteins)
PPP3CA, B, C	+ 2 regulatory subunits (PPP3R1, 2)
PPP4C	+ 3 regulatory subunits (PPP4R1, 2, 4)
PPP5C	- -
PPP6C	+ 6 regulatory subunits (PPP6R1-3, ANKRD proteins))
PPEF1	- -
PPEF2	- -

2. cysteine-based, Mg^{2+}/Mn^{2+} dependent phosphatases (PPM)
    - PPM1A-B, D-H, J-N
    - PDP1,2
    - PHLPP1, 2
    - ILKAP
3. aspartate-based protein phosphatases (CTD)
    - CTDNEP1
    - CTDP1
    - CTDSP1, 2
    - CTDSPL, L2
    - TOMM50
    - UBLCP1

FIGURE 18-2 Overview of the big family of protein phosphatases. *Information from the HUGO gene-nomenclature database.*

phosphates (PTP) and then focus on the functioning of class I members only. We then discuss the characteristics of protein serine/threonine phosphatases (PPP, PPM, and CTD) and provide a number of examples of action of members of the PPP subfamily only.

PROTEIN TYROSINE PHOSPHATASES

A soluble protein phosphatase specific for phosphotyrosine was first isolated from human placenta and named PTP1B (gene name PTPN1) (Tonks et al., 1988b). Its amino-acid sequence has stretches homologous with the tandem-repeat domains present in the cytoplasmic portion of the leukocyte common antigen CD45 (Charbonneau et al., 1989), a receptor-like protein highly expressed on the surface of cells of hematopoietic lineage. This discovery revealed the catalytic activity of CD45, which we now know is a receptor-like protein tyrosine phosphatase (gene name PTPRC). Using the DNA sequence that codes for the catalytic domain as a probe (the conserved "signature motif," (I/V)HC-X_5-R), some 113 distinct vertebrate PTP catalytic domains have been identified, with about 40 distinct classical PTP genes in the human genome. Of these classical PTP genes, 19 code for non-receptor tyrosine-protein kinases (designated as PTPN1, 2, 3, etc.) and 21 for receptor-like phosphatases (designated as PTPRA, B, C, etc.) (Figure 18-2, -3, and -4). The diversity of classical tyrosine-protein phosphatases is greatly enlarged through alternative splicing, alternative promoter usage, and posttranslational modification. Notably, as a result of alternative splicing, some of the PTPR subtypes also contain cytosolic variants. For example the isoform-2 and -3 of PTPRE, produced by alternative promotor usage and alternative initiation, respectively, are cytosolic. Isoform-2 acts as a negative regulator of insulin-receptor signaling in skeletal muscle and isoform-1 and -2 inhibit the response initiated by the high-affinity immunoglobulin epsilon receptor (FCER1A) (Andersen et al., 2001). A set of unrelated and rather diverse phosphatases, the dual-specificity phosphatases (DUSP), are incorporated in the family of protein tyrosine phosphatases. Since the first of the DUSP to be identified was VH1, encoded by vaccinia virus, they are sometimes referred to as VH1-like phosphatases (Guan et al., 1991).

Four distinct sets of protein tyrosine phosphatases are recognized (Figure 18-2). Class I covers both the classical and DUSP, each of which are further subdivided. The other three classes have very few members and we only treat them briefly below. Class II is represented by a single gene, ACP1, an acid phosphatase with a very low molecular weight. It counts 158 amino acids which indeed is small because a PTP-phosphatase domain already counts 275 amino acids (so not only a small protein but also a small phosphatase domain). It associates with clinical manifestations of diabetes mellitus: low activity protects against excesses of hyperglycemia. Class III phosphatases, represented by CDC25A, -B, and -C, function as dosage-dependent inducers of mitotic progression. CDC25A and CDC25, but not CDC25C, have been shown to play a role in the timing and efficiency of CDK1/cyclinB complex formation. They dephosphorylate the phosphotyrosine-15 residue of CDK1 and this facilitates complex assembly and stimulates kinase activity (under the condition where threonine-161 is phosphorylated by CDK7). The action of CDC25 is opposed by the serine/threonine kinase WEE1 (McGowan and Russell, 1993; Timofeev et al., 2010). The fourth group of phosphatases is represented by four homologues of the *Drosophila* eye-absent gene (EYA1-4). Both in *Drosophila* and in vertebrates, eye-absent genes play an essential role in diverse developmental processes among which, as their name suggests, eye development (Zimmerman et al., 1997). They catalyze the dephosphorylation reaction with an aspartate rather than a cysteine. They function both as protein phosphatase and transcriptional co-activators for the homeobox gene SIX1 (sine oculis homeobox homologue-1). Mutations in either SIX1 or EYA1 cause BOR (branchio-oto-renal) syndrome, an autosomal-dominant developmental disorder characterized by branchial fistulae, hearing loss, and renal anomalies (MIM entry 113650). SIX1 is normally fully down-regulated once organ development is completed. Its expression is low or undetectable in most normal adult tissues. However, re-expression of SIX1 occurs in cancer and together with EYA2 they promote epithelial–mesenchymal transition thereby augmenting cell dissemination (metastasis) (Patrick et al., 2013).

Web resources

- The Cold Spring Harbor Laboratory, in collaboration with Novo Nordisk Science, maintains a web-accessible resource of information on protein tyrosine phosphatases. URL: http://ptp.cshl.edu or the parallel site http://science.novonordisk.com/ptp.
- Consult the HUGO-Gene Nomenclature database for more detail about the members of the human PTP family. URLs: for non-receptor PTPN http://www.genenames.org/cgi-bin/genefamilies/set/812, for receptor PTPR http://www.genenames.org/cgi-bin/genefamilies/set/813.

Class I tyrosine phosphatases

Classical protein tyrosine phosphatases: non-receptor and receptor-like phosphatases

The non-receptor, or cytosolic, classical protein tyrosine phosphatases distinguish themselves by the possession of different accessory domains (Figure 18-3). The first to be discovered, PTPN1, comprises only a catalytic domain but all the others carry protein–protein interaction domains which play a role in subcellular localization of the enzymes. We mention PTPN6 and PTPN11, which carry two SH2 domains each through which they are recruited to phosphotyrosine peptides and thus recruit the phosphatases to subcellular locations where tyrosine kinases operate.

The receptor-like protein tyrosine phosphatases (PTPR) are transmembrane proteins and nearly all contain a tandem phosphatase domain, D1 and D2, both of which express the catalytic signature motif (Figure 18-4).

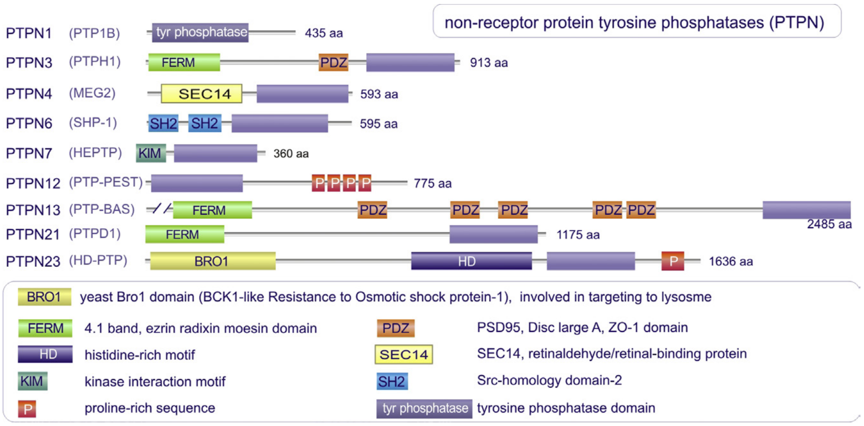

non-receptor protein tyrosine phosphatases (PTPN)
PTPN1 (PTP1B)
tyr phosphatase
435 aa
PTPN3 (PTPH1)
FERM
PDZ
913 aa
PTPN4 (MEG2)
SEC14
593 aa
PTPN6 (SHP-1)
SH2
SH2
595 aa
PTPN7 (HEPTP)
KIM
360 aa
PTPN12 (PTP-PEST)
P
P
P
P
775 aa
PTPN13 (PTP-BAS)
FERM
PDZ
PDZ
PDZ
PDZ
PDZ
2485 aa
PTPN21 (PTPD1)
FERM
1175 aa
PTPN23 (HD-PTP)
BRO1
HD
P
1636 aa
BRO1 yeast Bro1 domain (BCK1-like Resistance to Osmotic shock protein-1), involved in targeting to lysosme
FERM 4.1 band, ezrin radixin moesin domain
HD histidine-rich motif
KIM kinase interaction motif
P proline-rich sequence
PDZ PSD95, Disc large A, ZO-1 domain
SEC14 SEC14, retinaldehyde/retinal-binding protein
SH2 Src-homology domain-2
tyr phosphatase tyrosine phosphatase domain

FIGURE 18-3 Domain architecture of some non-receptor tyrosine phosphatases (PTPN). *Information from Andersen et al. (2001) and the HUGO gene-nomenclature database.*

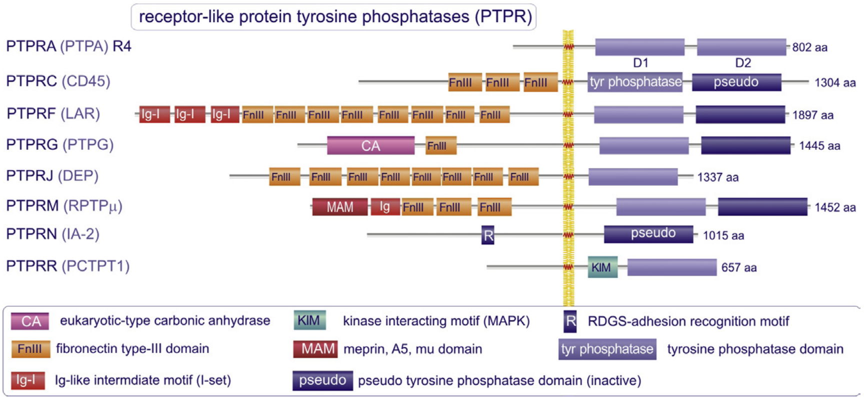

receptor-like protein tyrosine phosphatases (PTPR)
PTPRA (PTPA) R4
PTPRC (CD45)
PTPRF (LAR)
PTPRG (PTPG)
PTPRJ (DEP)
PTPRM (RPTPμ)
PTPRN (IA-2)
PTPRR (PCTPT1)
D1
D2
tyr phosphatase
pseudo
Ig-I
FnIII
CA
MAM
Ig
R
KIM
802 aa
1304 aa
1897 aa
1445 aa
1337 aa
1452 aa
1015 aa
657 aa
CA eukaryotic-type carbonic anhydrase
FnIII fibronectin type-III domain
Ig-I Ig-like intermdiate motif (I-set)
KIM kinase interacting motif (MAPK)
MAM meprin, A5, mu domain
pseudo pseudo tyrosine phosphatase domain (inactive)
R RDGS-adhesion recognition motif
tyr phosphatase tyrosine phosphatase domain

FIGURE 18-4 Domain organization of receptor tyrosine phosphatases (PTPR). All contain a tyrosine-protein phosphatase motif, often two, D1 and D2, in tandem, and are distinguished by their extracellular domains. Some have elaborate extracellular structures resembling adhesion molecules or growth-factor receptors, others appear rudimentary and ligand association is hard to imagine.

However, only the membrane proximal D1 domains are catalytically active. The inactivity of the D2 domains can be ascribed to the absence of invariant amino acids (tyrosine and asparagine, see next section) that converge around the active site. The preservation of the D2 domain among species and within nearly all receptor-like PTPs indicates that it probably serves an important physiological function. Its structural integrity is important for stability of the PTPR as a whole. Also, there is the possibility that D2 might be involved in the regulation of D1 or that it could assist in substrate recognition.

The PTPR family was initially classified on the basis of the composition of their extracellular segments (Fischer et al., 1991). Remarkably, when sorted according to the sequence homology of their catalytic domains, the classification remains almost the same. (The initial classification has therefore been retained.) The extracellular segments range from very short chains having no apparent function, to extended structures with putative ligand-binding domains similar to those present in adhesion molecules (fibronectin repeats or immunoglobulin repeats, Figure 18-4). Such diversity suggests a wide range of biological functions, which however, have proved hard to pin down. This is largely because identification of their physiological substrates has been hampered by the nonspecificity of these enzymes when assayed for activity in vitro. Moreover, although some are predicted to be receptors, their ligands have so far been elusive (Sap et al., 1994; Brady-Kalnay and Tonks, 1994).

Tyrosine specificity and catalytic mechanism

The classical protein tyrosine phosphatases are without effect on phosphoserine or phosphothreonine residues but there are a large number of phosphotyrosine proteins that act as substrates. This suggests that the overall structure of the substrate is not the main determinant of selectivity; instead substrate recognition is primarily determined by the presence of the phosphotyrosine residue in the context of its peptide environment. In particular, specificity results from the depth of the catalytic cleft (Figure 18-5).

Unlike the kinases (extensively discussed in Chapter 2, “An Introduction to Signal Transduction,” section “Protein kinases catalyze the phosphate transfer”), the catalytic domains of tyrosine-protein phosphatases do not require posttranslational modifications in order to become catalytically competent. The binding of the substrate induces a conformational change in the protein in which the WPD loop closes around the substrate to create the recognition pocket and generates the catalytically competent form of the enzyme (Figure 18-5, panel (a)). Protein tyrosine phosphatases are however, subject to regulation, either by intramolecular interactions, by phosphorylation of noncatalytic domains, by association of regulatory subunits, or by spatial separation from substrate.

While the ternary structure of classical PTP phosphatases is highly conserved, the surfaces are surprisingly diverse. Mapping of surface residues identify only a few conserved surface patches comprising loop regions surrounding the active site. Conserved surfaces are formed by the phosphotyrosine loop (KNRY motif in purple in Figure 18-5) and by conserved

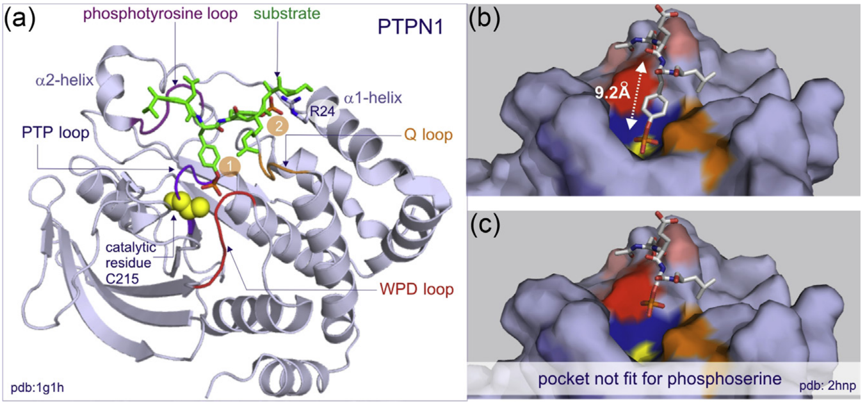


FIGURE 18-5 The arrangement at the active site of PTPN1 (PTP1B). (a) The sides of the catalytic cleft of classic tyrosine phosphatases are characterized by three motifs: (1) the WPD loop, containing an invariant Asp; (2) the Q loop, containing an invariant Gln; and (3) the phosphotyrosine loop, containing an invariant tyrosine. The catalytic residue is C215 (but here mutated into an alanine to make a catalytically dead mutant). The phosphatase harbors two phosphotyrosine-binding sites the first in the catalytic pocket and the second at the loop connecting the α1- and α2-helices (second-site loop). Arginine-24 (R24) plays an important role in coordinating the negatively charged phosphate group. (b) The invariant tyrosine in the phosphotyrosine loop determines the depth of the catalytic cleft, approximately 10 Å, and allows phosphotyrosine to make contact with the catalytic cysteine residue (yellow). (c) The pocket is too deep for phosphoserine or phosphothreonine to contact the catalytic cysteine residue.

glutamine residues of the Q loop (light orange). Conserved WPD loop (red) residues are largely buried. Highly diverse regions, including the two residues following the KNRY motif and the topology of the secondary substrate-binding pocket (loop following the α1-helix) contribute to defining substrate specificity. The diverse PTP surfaces give rise to significant differences in surface electrostatic potential, a property that is likely to influence substrate recognition, association with regulatory proteins, and regulatory mechanisms such as dimerization (Barr et al., 2009).

The catalytic mechanism of the tyrosine phosphatases is quite different from that of the serine/threonine phosphatases. These are metalloenzymes that dephosphorylate their substrates in a single reaction step involving a metal-activated nucleophilic water molecule. In contrast, the classical tyrosine phosphatases catalyze dephosphorylation through a cysteinyl-phosphate enzyme intermediate (reviewed in Tonks, 2003) (Figure 18-6).

Classical PTP phosphatases in signal transduction

At first, interest in protein tyrosine phosphatases was driven by the perception that they might be antitumorigenic. They appeared to offer the possibility of counteracting the transforming effects of mutated and, therefore, constitutively activated protein tyrosine kinases and were considered as possible tumor suppressors. Thus it came as quite a surprise when it was found that some classical PTP phosphatases, rather than opposing the actions of the kinases, actually cooperate with them to reinforce their signals. As an example, overexpression of a receptor-like protein tyrosine phosphatase, RPTPA, in rat embryo fibroblasts

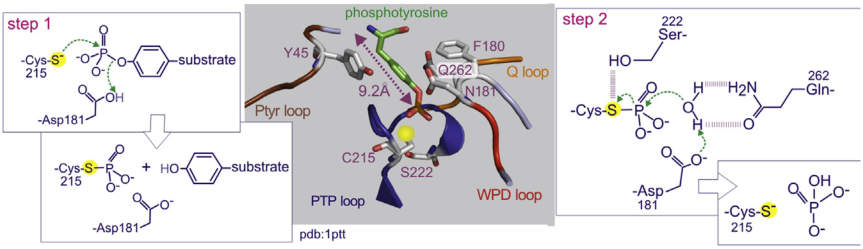


FIGURE 18-6 Catalytic mechanism of classical tyrosine phosphatases (PTPN1). In the first step, there is transfer of an electron from Cys-215 to the substrate phosphate. The catalytic cysteine has a low pKa (5.4) so that the sulfydryl group acts as a nucleophile ($-S^{-}$). This is coupled with protonation of the tyrosyl-leaving group by the side chain of the conserved Asp181, leading to the formation of a cysteinyl-phosphate intermediate. In the second step involving Gln-262 and Asp-181 there is hydrolysis of the catalytic intermediate with release of phosphate. Cysteine and aspartate make up for the highly conserved motif of protein tyrosine phosphatases C(X5)D. The placement of the essential residues at the active site is depicted in the middle panel. Note that the phosphotyrosine substrate is aligned and sandwiched between Y45 and F180. Y45 determines the depth of the catalytic cleft and is the major determinant of specificity for phosphotyrosine.

causes persistent activation of SRC with concomitant cell transformation (Zheng et al., 1992). In accordance with this, increased RPTPA mRNA levels have been detected in late-stage colorectal tumors and enhanced levels of the enzyme occur in one-third of breast carcinomas. However, overexpression of PRPTPA in breast carcinoma cells actually reduces tumor aggressiveness. The functional significance of PRPTPA thus depends on the cellular context and the type of tumor. With respect to a possible role in cancer, 19 PTP genes map to chromosomal regions frequently deleted in human cancers and four PTP genes map to regions frequently amplified in human cancers. Below we consider the different roles of classical protein tyrosine phosphatases on the basis of a number of examples.

Classical non-receptor PTP phosphatases

PTPN1, diabetes, and obesity Perhaps the most spectacular example of a link between a PTP and human disease is type II (mature onset, insulin-resistant) diabetes and obesity. Both genetic and biochemical studies provide good evidence for a role of tyrosine phosphatase in the signaling events downstream of the insulin and leptin receptors (Kenner et al., 1993). Dephosphorylation of the insulin receptor by protein tyrosine phosphatases is critical in the control of the cellular response to insulin. Numerous studies have demonstrated that in humans, and in animal models, the resistance to insulin in type II diabetes and obesity is accompanied by increases in PTP activity and increases in the level of expression of defined members of the PTP family, PTPRF (or LAR), and PTPN1 (or PTP1B) (Ahmad et al., 1997). However, disruption of the PTPRF gene in mice yields a complex phenotype consistent with having a postreceptor defect in insulin signaling but, surprisingly, associated with impaired activation of downstream signals such as PI3-kinase (rather than the expected elevated signal). Matters are somewhat clearer for PTPN1. Here, insulin-induced activation of ribosomal S6 kinase (protein synthesis) and transition through the G2/M phase checkpoint of the cell cycle is inhibited in *Xenopus* oocytes injected with the purified enzyme (Tonks et al., 1990; Cicirelli et al., 1990). When overexpressed in Rat1 fibroblasts, PTPN1 reduces insulin-induced phosphorylation of its own receptor, the downstream phosphorylation of components of the signaling cascade, and translocation of SLC2A4 (glucose transporter-4) to the membrane and glycogen synthesis (Chen et al., 1997). Conversely, loading of hepatoma cells with neutralizing antibodies to PTPN1 enhances insulin-induced phosphorylation of its receptor and of the receptor substrate IRS1 (Figure 18-7).

An unequivocal link between insulin signaling and the tyrosine phosphatase was established through the use of PTPN1 knockout mice: these animals remained healthy and displayed an enhanced sensitivity to insulin

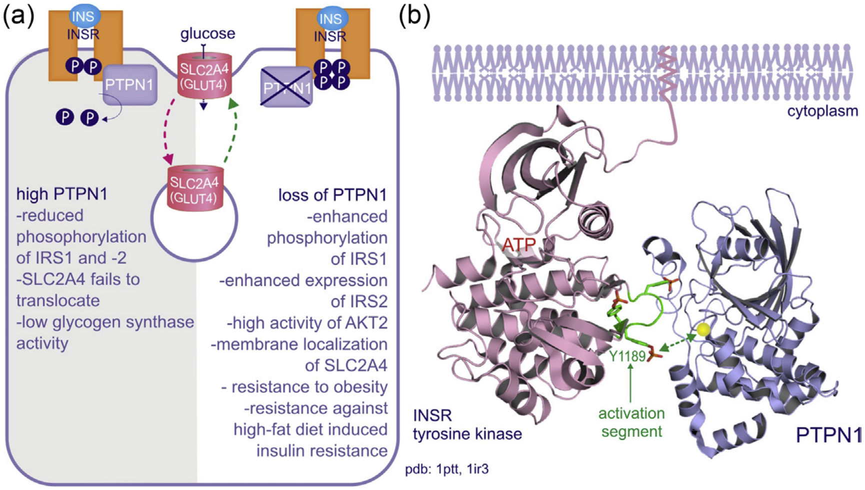


FIGURE 18-7 PTPN1 down-regulates insulin signaling by dephosphorylation of the receptor and its substrates (IRS). (a) PTPN1 inhibits the action of insulin by dephosphorylating the cytosolic segment of the insulin receptor. Lack of PTPN1 activity promotes glucose uptake, due to elevated cell surface expression of its transporter, but (in rats, anyway) it also protects against the effects of high-fat diet-induced insulin-resistance and obesity. (b) PTPN1 dephosphorylates preferentially the bis- or tris-phosphorylated activation segment of the insulin receptor (Salmeen et al., 2000). Residue pTyr-1189 is most readily recognized by the catalytic cleft of the phosphatase. Dephosphorylation renders the insulin receptor catalytically incompetent. Sequence comparison with other PTPs suggests that the features that confer the specificity of this reaction are unique to PTPN1 and its close relative PTPN2 (TC-PTP).

(Elchebly et al., 1999). Moreover, when fed a high-fat diet they failed to become obese and retained their normal sensitivity to insulin. By contrast, their wild-type (PTPN1$^{+/+}$) littermates suffered rapid weight gain (eventually becoming truly obese) and onset of insulin resistance which coincided with a reduced level of tyrosine phosphorylation of the insulin receptor (Klaman et al., 2000). All this suggests that the insulin receptor intracellular segment acts as a substrate of PTPN1. Indeed PTPN1 has been reported to associate with the INSR kinase domain, however, not to the site that exposes the phosphotyrosines (activation segment) but to the opposite sites (Li et al., 2005). Receptors often occur in clusters so perhaps that PTPN1 bound to one insulin receptor dephosphophorylates another.

Surprisingly, the knockout mice did not show any predisposition to cancer despite the potential of PTPN1 to regulate growth factor-receptor tyrosine kinase signaling and to counteract the transforming effects of the kinases SRC and Neu (ERBB2 of EGFR) (Dixon and Rhodes, 1992; Bruskin et al., 1992). The activity of MAPK3 (ERK2) present in fibroblasts obtained from these mice was only slightly enhanced and there was no effect on the activation level of AKT2. A possible explanation comes from the finding that, due to a lack of PTPN1, expression of the protein RASA1 (Ras GTPase activating protein possessing an SH2 domain) is elevated and that its membrane-recruiting partner, DOK1, is highly phosphorylated (offering SH2-binding sites). Bringing RASA1 to the membrane results in the attenuation of the RAS–MAPK pathway because of the enhanced hydrolysis of GTP (Carpino et al., 1997; Dube et al., 2004). This is a good example of how feedback mechanisms prevent excess signaling (often referred to as the "robustness of the system").

PTPN1 as a possible therapeutic target for the treatment of type II diabetes and obesity? Introduction of antisense oligonucleotides to PTPN1 into obese (leptin-deficient mice, "ob/ob") and late onset diabetic (leptin-receptor-deficient mice, "db/db") mice has the effect of suppressing PTPN1 gene expression in the liver and adipose tissue, but not in muscle. The mice exhibit enhanced sensitivity to insulin but maintain normal levels of blood glucose (Zinker et al., 2002). This, and the resistance to obesity, has made PTPN1 an attractive target for treating type II diabetes as well as obesity in general. The quest for inhibitors of PTPN1 has attracted much attention and more than 120 crystal structures of complexes of PTPN1 with inhibitors or substrates have been deposited in the Protein Data Bank. Numerous therapeutic agents have been developed but none have yet reached medical approval (Thareja et al., 2012).

The leptin receptor is a cytokine receptor. It resembles the CSF2 (GM-CSF) receptor and signals mainly through the JAK2/STAT3 pathway. It also activates the IRS2/PI 3-kinase and RAS/MAPK pathways. CSF2 (GM-CSF): granulocyte–monocyte colony stimulating factor or colony-stimulating factor-2.

Insulin and leptin act on the neurons in the hypothalamic arcuate nucleus to regulate the desire to imbibe food. The two hormones signify positive energy balance and satiety, manifested by (among other changes) increased release of proopiomelanocortin (POMC) peptides and reduced release of neuropeptide Y. The consequence is suppression of appetite and a greater propensity to undertake physical activity. In contrast, depletion of fat stores gives rise to low basal insulin and low leptin levels, which favor food intake and economy of energy expenditure. Disruption of insulin and/or leptin signaling encourages food intake and suppresses energy expenditure, hence, an increase in body weight. Stimulating insulin and/or leptin signaling does the reverse. The factors perturbing the balance giving rise to the onset of obesity is the focus of intense research.

Optimal substrate recognition by PTPN1 requires the amino-acid motif: D/E-pY-pY-R/K, is present not only in the insulin receptor (-D-pY-pY-R-) but also in the receptor tyrosine kinases of the FGF- and NGF-receptor, and in the tyrosine kinases NTRK1, AXL, and, of particular relevance here, in members of the JAK family (Janus kinase) (Touw et al., 2000). With respect to obesity, the JAK kinases are instrumental in signaling by the satiety hormone leptin via its receptor OB-R_L (for review, see Tartaglia, 1997). JAK2, a physiological substrate of PTPN1, phosphorylates two residues on the human leptin receptor LEPR (formerly OB-R) and acts to stimulate leptin signaling by promoting the binding and subsequent phosphorylation and activation of the STAT3 transcription factors. In the POMC neurons of the arcuate nucleus (part of the hypothalamus), STAT binding to DNA enhances expression of the large precursor protein POMC, from which αMSH is cleaved (short peptide acting as neurotransmitter). A high content of αMSH and subsequent elevated level of release of αMSH at synapses that connect POMC with anorexigenic neurons (situated in the paraventricular nucleus) contributes to the sentiment of satiety. Hypothalamic neurons derived from PTPN1 knockout (PTPN1$^{-/-}$) mice display markedly increased leptin-induced STAT3 phosphorylation (Figure 18-7). Since leptin-activated JAK2 is a substrate of PTPN1 but not STAT3 or the leptin receptor itself (Flint et al., 1997) it follows that PTPN1 negatively regulates leptin signaling through inhibition of JAK2, and provides a mechanism by which it may regulate obesity (the animal remains hungry) (Flint et al., 1997; Cheng et al., 2002).

The positive action of STAT3 is counteracted by the transcription factor FOXO1 (Ma et al., 2015). A strong response thus necessitates its removal from the nucleus. Here is where insulin comes into play. Through its action on the insulin receptor it raises the activity of the protein kinase AKT2, which phosphorylates and sequesters FOXO1 in the cytoplasma (attached to the phosphoserines-binding protein 14-3-3) (Kitamura et al., 2006). Mice with specific depletion of FOXO1 in the POMC neurons appear resistant to diet-induced obesity (Plum et al., 2009) (Figure 18-8).

POMC stands for proopiomelanocortin, a larger precursor protein from which 11 short peptides are cleaved. These are NPP, melanotropin-γ, potential peptide, corticotropin, melanotropin-α (αMSH), cotricotropin-like intermediate peptide, lipotropin-β, lipotropin-γ, melanotropin-β, β-endorphin, and met-enkephalin. For more information refer to UniProt entry P01189.

AGRP, agouti-related protein, acts as an inverse agonist of αMSH. It means that it binds the MC4R receptor, but drives it into the inactive state. A high production of AGRP causes inhibition of anorexigenic neurons because of the loss of stimulation by αMSH. For more information about reverse-agonists, return to Chapter 3, "Regulation of Muscle Contraction by Adrenoceptors," Figures 3-8 and 3-9.

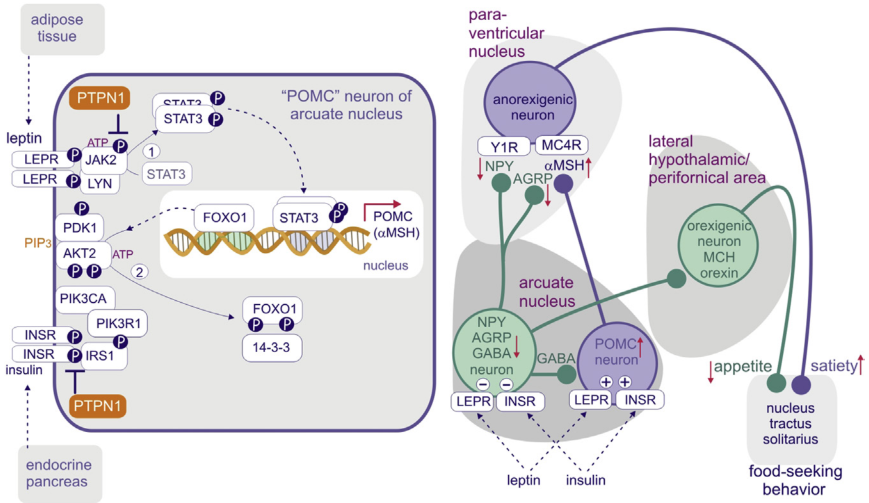


FIGURE 18-8 Role of PTPN1 in satiety signaling in the arcuate nucleus. High fat (or low fat turnover) releases leptin, a satiety signal, from adipose tissue. This binds to its receptor, LEPR, in neurons of the arcuate nucleus of the hypothalamus, and activates JAK2. This phosphorylates STAT3, which dimerizes and enters the nucleus, there to drive expression of POMC, the large precursor protein from which numerous small peptides are cleaved (among which αMSH). However, the action of STAT3 is abrogated by FOXO1 and a second signal is necessary to remove this transcription factor from the nucleus. Here is where insulin contributes to the action of leptin. It activates the PI -3kinase/AKT2 pathway which results in the phosphorylation and cytoplasmic sequestration of FOXO1. Cells harboring an activated JAK2/STAT3 pathway secrete predominantly αMSH, CART, and CRH and this translates into loss of appetite (anorexic signal) and energy-spending behavior. αMSH stimulates anorexigenic neurons in the paraventricular nucleus and these then will dominate in the decision process at the level of the nucleus tractus solitarius ("do I get up and seek food or not"). PTPN1 reduces both the leptin and insulin signal and thus reduces the sense of satiety, as a consequence it favors appetite.

Redox regulation of PTPN1: reactive oxygen species as second messengers Numerous cells, including phagocytes, epidermal cells, fibroblasts, vascular smooth muscle cells, and osteoblasts, generate reactive oxygen species (ROS) in response to environmental cues. ROS originate from two different sources. The first concerns production by the NADPH oxidase, NOX1, a pyridine nucleotide-dependent oxidoreductase (that also operates as voltage-gated proton channel). It resembles the type b-cytochrome heavy chain (gp91phox, gene name CYBB) present in phagocytes. The NADPH oxidase transfers electrons, across the plasma membrane delivering them to the acceptor O_2, generating superoxide (O_2^-) (Figure 18-9). TGFβ1, IL1A/B, TNF, insulin, PDGF, EGF, angiotensin, thrombin, or lysophosphatidic acid are among the cytokines that activate this pathway (Ohba et al., 1994; Sundaresan et al., 1995; Irani et al., 1997). For a review see Chiarugi and Cirri (2003).

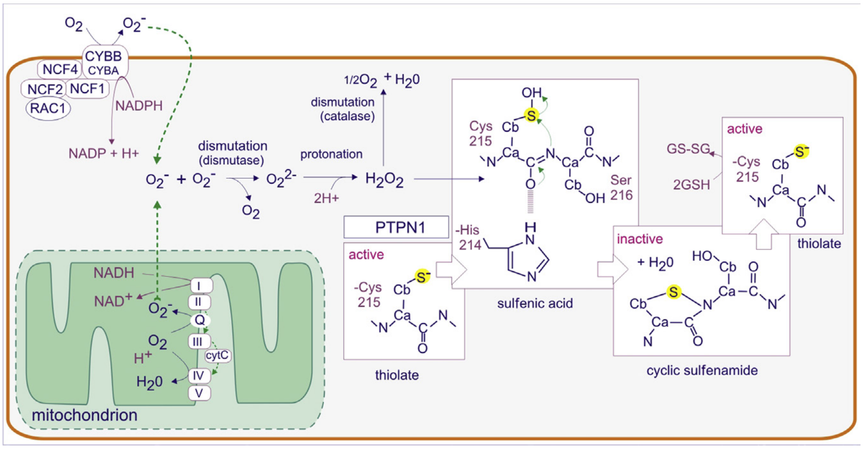


FIGURE 18-9 Generation of superoxide (O_2^-) by NADPH oxidase and the mitochondrial electron transport chain. Production of superoxide (O_2^-) occurs through activation of NADPH oxidase (NOX1), a pyridine nucleotide-dependent oxidoreductase. It is also released from mitochondria. Superoxide is converted to oxygen and peroxide (O_2^{2-}), which is protonated to produce H_2O_2. This causes the formation of a cyclic sulfonamide unable to launch a nucleophilic attack on tyrosine phosphate.

A second source of ROS, operational in all cell types, is the mitochondrial electron transport chain. Here, superoxide is released at the level of ubiquinone (which normally shuttles electrons from complex II to III). This plays a role in apoptosis, but transient production also occurs in response to other cues under conditions where apoptosis does not occur. Examples are hypoxia, the expression of oncogenic RAS or MYC, the presence of cytokines such as TNF and PDGF, or the interaction of integrins with the extracellular matrix (Figure 18-9). The mechanism by which this ubiquinone pathway is activated remains to be elucidated (Werner and Werb, 2002; Vafa et al., 2002). Superoxide is short lived, being dismutated into molecular oxygen (O_2) and peroxide (O_2^{2-}), which as H_2O_2, is converted by catalase into oxygen and water. ROS, in particular H_2O_2, survive long enough to modify the active site cysteine residue of tyrosine phosphatases.

ROS as second messengers There have been a number of claims for a role of ROS, in particular H_2O_2, as intracellular messengers that regulate protein phosphorylation and gene expression. An early hint was provided by the observation that H_2O_2 can mimic the stimulatory effect of insulin on glucose transport and lipid synthesis in adipocytes (Mukherjee et al., 1978; May and de Haen, 1979). Later it was found that O_2^- can stimulate members of the MAP-kinase family (Baas and Berk, 1995) and that blunting the spike of H_2O_2 (using catalase or the antioxidant N-acetylcysteine) obscures a number of signal transduction events such as phosphorylation and activation of MAP-kinase in response to PDGF. The cells also fail to enter the S-phase of the cell cycle and lose their response to chemotactic stimuli (Sundaresan et al., 1995). Further evidence for a second messenger role of ROS came from experiments that showed that EGF-mediated autophosphorylation of its receptor and the subsequent phosphorylation of receptor substrates can be can be up-regulated by the addition of H_2O_2 to the culture medium. The enhanced phosphorylation can be prevented by intracellular microinjection of catalase. Several studies have demonstrated an inhibitory effect of H_2O_2 (at μM concentrations) on various tyrosine phosphatases, both membrane bound and cytosolic (Seo et al., 1997; Barrett et al., 1999; Blanchetot et al., 2002; Meng et al., 2002). Striking evidence for the role of NADPH oxidase in cellular signaling came from the finding that overexpression of NOX1 in NIH3T3 cells produces particularly aggressive tumors following injection into nude (athymic) mice. Sustained and elevated levels of hydrogen peroxide are required to maintain the transformed state (Lassegue et al., 1999; Arnold et al., 2001).

It is now widely accepted that ROS are transiently generated in cells in response to extracellular stimuli; and which act on effector systems can be regarded as second messengers. Oxidative modification of catalytic-site cysteine residues resembles the mode of action of phosphorylation (Figure 18-9). Receptor tyrosine kinase-induced generation of ROS causes prolonged inactivation of tyrosine phosphatases and so maintains the phosphorylated (and activated) state of the receptor (positive feedback loop) (Figure 18-10). Their brief generation-time ensures that over a period of about a few minutes the enzymes return to their reduced state, capable once again of dephosphorylating (and inactivating) the receptor tyrosine kinase and its substrates (Lee et al., 1998; Denu and Tanner, 1998; Goldstein et al., 2001).

PTPN6 and PTPN11, SH2 domain-containing protein tyrosine phosphatases PTPN6 (formerly SHP-1) and PTPN11 (formerly SHP-2) are two members of the non-receptor PTPs that contain two N-terminal SH2 domains. They bind to tyrosine-phosphorylated growth-factor receptors (EGFR, FGFR, HGFR (Met) or PDGFR), the interferon-α receptor (IFNAR1), scaffolding adapters (IRS1, -2 and GAB1), the ITAM motifs of the immune stimulatory receptors such as the CD3zeta chain (CD247), and the high-affinity immunoglobulin-ε receptor (FCER1A), and to the adaptor protein TYROBP (in hematopoietic cells). They also associate with immune inhibitory receptors, carrying ITIM motifs such as the low-affinity immunoglobulin-γ receptors (FCGR2A or -B) (Ravetch and Lanier, 2000).

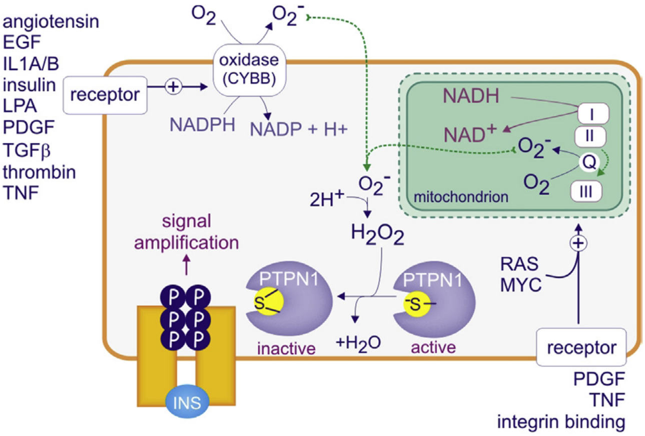


FIGURE 18-10 Stimulation of production of ROS by growth factors, hypoxia, oncogenes, and integrin binding. Various growth factors, cytokines, oncogenes, and integrins, as well as hypoxia, enhance production of reactive oxygen species. Hydrogen peroxide causes inactivation of the active-site cysteine of PTPN1. The transient inhibition enhances and prolongs phosphorylation of receptor tyrosine kinases and amplifies the growth-factor signal. Other targets of H_2O_2 are proteins having an acidic cysteine residue (pKa 5.4) such as other tyrosine phosphatases and the transcription factors NFKB and HIF.

The C-termini of PTPN6 and -11 contain two phosphorylation sites which are targets of the PDGF receptor or cytoplasmic kinases such as JAK or TYK2. These phosphotyrosine residues act as docking sites for other SH2 or PTB domain-containing proteins (Figure 18-11).

There are two vertebrate SHP genes, PTPN6 and PTPN11 and invertebrate orthologs such as Csw (corkscrew) in *Drosophila* and Ptp-2 in *C. elegans* (Neel et al., 2003). PTPN6 is mainly (but not exclusively) expressed in hematopoietic cells, PTPN11 is ubiquitously expressed. Despite the general similarity of PTPN6 and PTPN11, their functions are quite distinct. Whereas PTPN6 is generally inhibitory, PTPN11 acts as stimulator of the RAS–MAPK pathway.

Structural analysis of PTPN6 has revealed that the "backside" of the N-SH2 domain is wedged into the catalytic domain (Figure 18-11). This simultaneously obstructs the catalytic cleft and distorts the phosphotyrosine-binding site. In this configuration, called the basal state, activity is low. Binding of the N-terminal SH2 domain to a pY-containing peptide can relieve some of the intramolecular inhibition but full activity is only achieved when both SH2 domains are engaged (Hof et al., 1998). This occurs when PTPN6 binds to tandem phosphorylation sites on phosphorylated tyrosine kinase receptors, some of their substrates or tyrosine-phosphorylated cytokine receptors (Yang et al., 1998). The open conformation becomes stable when the N-SH2 folds back to a new position on the catalytic domain (Wang et al., 2011) (Figure 18-11(b)).

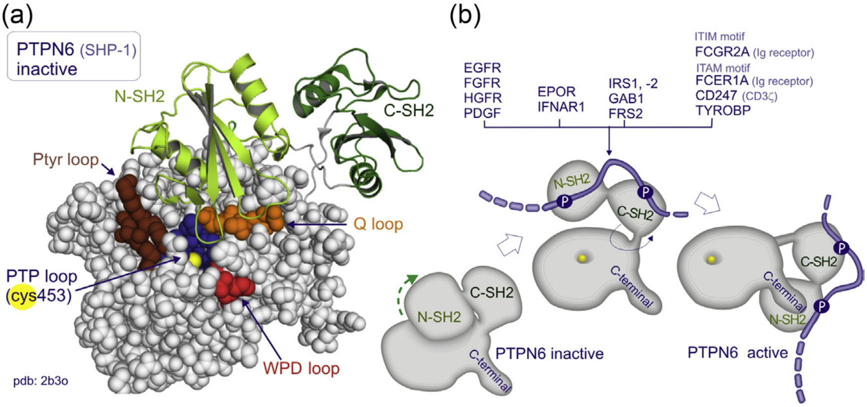


FIGURE 18-11 Autoinhibition of PTPN6 and -11. (a) The most N-terminal SH2 domain of PTPN6 wedges into the catalytic cleft of the tyrosine phosphatase domain. One of the loops of N-SH2 contacting the PTP-loop region to obscure the pocket that leads to the catalytic Cys residue (yellow). (b) When the SH2 domains are bound to tyrosine-phosphorylated proteins, the inhibitory restraint on SHP is relieved. Engagement of both SH2 domains induces maximal activity. Examples of tyrosine-phosphorylated proteins include receptors (EGFR, FGFR, HGFR, or PDGFR), adaptor proteins GAB1 and FRS2, and the tyrosine-phosphorylated ITIM, and ITAM motifs of immunoglobulin Fc receptors of CD247 (CD3ζ-subunit of the T-cell receptor) and of TYROBP (attached to diverse receptors).

PTPN6 and the control of inflammation A role for PTPN6 was first indicated in mice having a somewhat moth-eaten appearance with patches of inflamed skin and loss of hair (Tsui et al., 1993). This phenotype, arising from absence of PTPN6, is due to a systemic autoimmune condition caused by abnormalities in cells of hemapoietic lineage and it is marked by a general overexpansion of cell numbers. The differentiation and functions of natural killer cells and erythroid cells are also adversely affected. There is an accumulation of macrophages and neutrophils in the lungs and the mice die within weeks of birth. Two naturally occurring point mutations are at the origin of the moth-eaten (me) phenotype. The moth-eaten (me) allele generates an early frameshift and consequently the me/me mice are protein-null (lethal after weeks). The moth-eaten (viable, me^{v}) allele encodes two aberrant PTPN6 proteins that possess 20% of the normal enzyme activity (lethal after months) (reviewed in Neel et al., 2003). On this basis, PTPN6 can be regarded as a negative regulator of signaling.

Biochemical and functional studies established this role in a wide range of signaling pathways downstream of receptors for ligands that include EPO, prolactin, CSF1, EGF, BCR, and TCR (CD3ζ-subunit) (reviewed in Neel et al., 2003 and Zhang et al., 2000). There are multiple ways in which PTPN6 interferes but the common theme is that PTPN6 is invariably recruited into signaling complexes through SH2 domains. This brings the phosphatase into the proximity of the phosphotyrosyl groups of the receptors, recruited cytoplasmic kinases or the multiphosphorylated-docking proteins. Moreover, PTPN6 itself can be phosphorylated on its C-terminal tyrosine residues, which converts it into an adaptor. One of the proteins that bind to the phosphotyrosine residues is GRB2, also an adaptor, and via its SH3 domain it recruits SOCS1 into the complex. SOCS1, suppressor of cytokine signaling, is part of an E3-ligase complex that is composed of Cullin5 (CUL5), RBX1 and TCEB1, TCEB2 (also known as elongin proteins) (Okumura et al., 2012). SOCS1 plays the role of the receptor, bringing the E3-ligase complex to the targets. E2-ubiquitin conjugating enzyme, the ubiquitin donor, attaches to RBX1. Multiple ubiquitins are transferred to JAK1 and STAT5 proteins (both of which have numerous ubiquitinylation sites) and this leads to their demise. (Figure 18-12, panel (a)). For more information about ubiquitinylation, return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor and Signaling through Ubiquitinylation," essay: "Ubiquitinylation and SUMOylation."

A more selective gene knockout study, targeting exclusively naïve B-cells, showed that PTPN6 plays a role in the migration of antibody-secreting B-cells. Naïve B-cells, after having encountered specific antigens and with

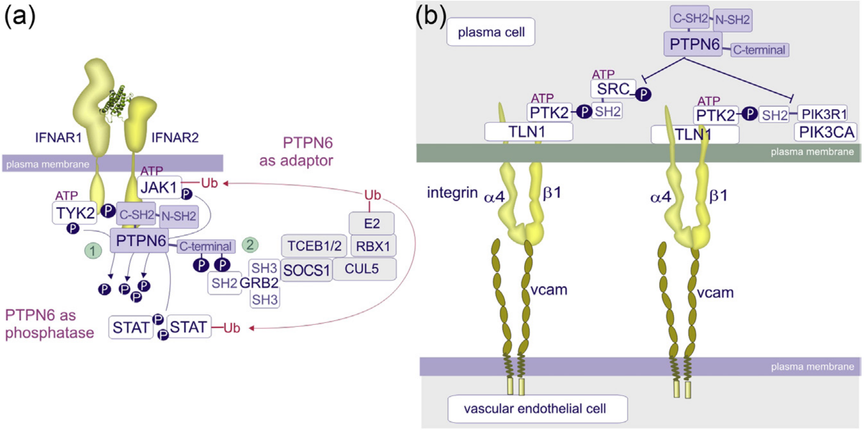


FIGURE 18-12 PTPN6-mediated inhibition of receptor functions. (a) Direct recruitment of PTPN6 to cytokine or growth-factor receptors (1) causes dephosphorylation in the activation segment and inactivation of JAK2. This is followed by dephosphorylation of STAT transcription factors and of the receptor. Tyrosine-phosphorylated PTPN6 can also act as an adaptor to recruit GRB2/SOCS1 into the receptor-signaling complex (2). SOCS1 in turn, recruits an E3-ubiquitin ligase complex to the cytokine receptor and sets in train the ubiquitylation and subsequent destruction of JAK and STAT. (b) Studies on antibody-secreting plasma cells have shown loss of PTPN6 causes an increase in signaling from adhesion complexes as measured by an enhanced phosphorylation of SRC and PI3-kinase (PIK3CA/PIK3R1). This may prevent a cyclic activation/inactivation of α4β1 integrins and thus prevent migration over a VCAM substrate.

the help of follicular T-helper (T_{FH}) cells, turn into antibody-secreting plasma cells and memory B-cells (McHeyzer-Williams et al., 2012; Tarlinton et al., 2008). They migrate to the bone marrow where they occupy protective "survival niches" and this allows both antibody-secreting plasma- and memory-B-cells to establish a long-lived pool (carriers of humoral immunity). The loss of PTPN6 does not impair differentiation toward antibody-secreting cells but does prevent homing to the protective bone-marrow niches. The affected mice therefore lack plasma and memory B-cells and, as a consequence, fail to establish life-long humoral immunity (Li et al., 2014). Integrins α4β1 and αLβ2 play an important role in the migration from the germinal centers in the peripheral lymph nodes to the bone marrow. Return to Chapter 14, "Chemokines and Traffic of White Blood Cells," for more information about the role of integrins in trafficking of white blood cells. PTPN6-deficient B-cells display limited migration across wells coated with VCAM1. The underlying reason is that the cells show a higher avidity for VCAM1 and they simply fail to detach thus impeding movement over the substrate. The on/off state of integrin adhesion molecules, in particular integrin α4β1 in relation to VCAM1, is essential for cell motility (Roach et al., 1998; Abram et al., 2013). Loss of PTPN6 causes an excessive activation of the tyrosine-protein kinase SRC and PI3-kinase, both signaling components of integrin-based focal adhesion contacts in which tyrosine phosphorylation plays a key role (Li et al., 2014). How PTPN6 is recruited and which substrates are targeted is not yet clear (Figure 18-12(b)).

PTPN6 also enhances the sensitivity of cells to apoptosis. Neutrophils from mice possessing just one *me* allele ($me^{+/-}$) are more resistant to apoptosis and this might contribute to the increased number of white blood cells in the moth-eaten phenotype. PTPN6 binds to the receptors FAS and TNFRSF1A, both of which contain a conserved cytoplasmic phosphorylation motif (in the death-domain) that is recognized by PTPN6, causing its activation. Activated PTPN6 then sensitizes the cell for apoptosis, possibly by counteracting the protective activity of the kinase LYN (Daigle et al., 2002). It should be noted that phosphorylation of both FAS and TNFRSF1A may only occur when the receptor is not complexed with death-domain adaptors (TRADD and RIPK1 proteins) (Figure 18-13).

PTPN11 potentiates the RAS–MAP-kinase pathway Targeted disruption of the gene encoding PTPN11 in mice causes early embryonic death, but from this, other than concluding that it is important, little can be learned. Early indications about a possible role of PTP11N in cell signaling came from three lines of evidence.

First, the gene is homologous to *Drosophila* Corkscrew (csw). The corkscrew pathway directs the differentiation of the terminal, non-segmented regions of the fly embryo (Perkins et al., 1992). Csw is involved in activation of the serine/threonine kinase phl (equivalent to human RAF).

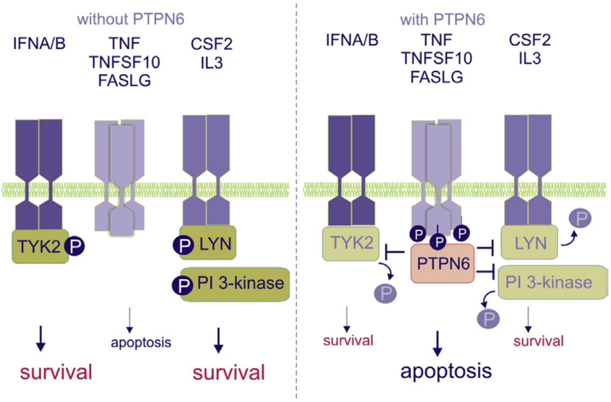


FIGURE 18-13 PTPN6 in sensitization of neutrophils to apoptosis. PTPN6 binds to tyrosine-phosphorylated FAS or TNFRSF1A receptors and then interferes with LYN- and TYK2-mediated tyrosine phosphorylation of downstream effectors. This way it blocks survival signals and renders neutrophils more sensitive to apoptosis.

Phl is activated through the Ras pathway (ras85D) and this implies that the phosphatase provides a positive signal downstream of receptor tyrosine kinase activation. Molecular and genetic analyses indicate that the dos protein is a likely substrate (Raabe et al., 1996). This contains a PH domain which permits membrane association, and numerous tyrosine-phosphorylation sites through which it could act as a membrane-bound docking protein involved in relaying signals from sevenless (sev, an insulin-like R-PTK) to ras85D (return to Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases,” Figure 10-13).

GAB1, a human protein that resembles dos, is also associated with PTPN11. In PTPN11$^{-/-}$ embryonic stem cells there is an enhancing effect on EGF signaling which requires the concerted action of GAB1 and an unidentified tyrosine-phosphorylated protein p90 (Figure 18-14) (Shi et al., 2006).

Secondly, expression in *Xenopus* embryos of a mutant form of PTP11N lacking the catalytic domain, causes tail truncations and prevents animal cap elongation (*short toad*, composed mainly of head structures) (Tang et al., 1995). These processes are determined in part by the receptor for FGF which also signals through the MAP-kinase pathway and so the phosphatase PTPN11 must act between the FGFR and the activation of MAP kinase. Similarly, fibroblasts expressing the catalytically inactive form of PTPN11, fail to activate MAP kinase in response to FGF, PDGF, or insulin-like growth factor (IGF) (Shi and Lu, 1998).

Thirdly, in mice, there is an essential role for PTPN11 in limb development and in branchial (pharyngeal)-arch formation, two other pathways controlled by FGFR signaling (Saxton et al., 2000).

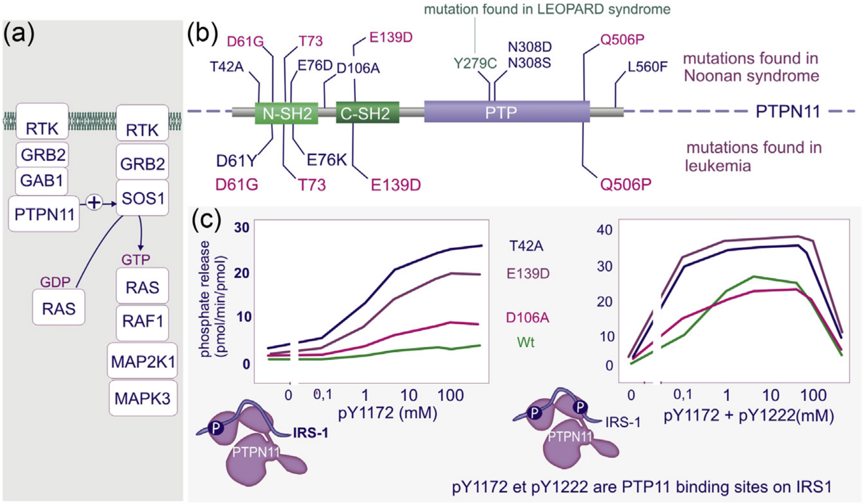


FIGURE 18-14 PTPN11 and its role in activation of the RAS–MAP-kinase pathway. (a) Genetic and biochemical analysis place PTPN11 between SOS1 and RAS. It acts on SOS1, probably by rendering the CDC25 domain (GEF domain) accessible, but details of the mechanism by which this happens are still lacking. (b) Mutations in PTPN11 are linked with the Noonan syndrome and with leukemia. In green a mutation that is associated with the LEOPARD syndrome. (c) Note that mutations T42A, D106A, and E139D facilitate activation of the phosphatase (gain-of-function mutations). This is the consequence of a weaker interaction with the N-terminal SH2 domain, which normally acts as an inhibitory subunit that needs to be removed through binding to phosphotyrosines. Also note that when phosphoproteins can engage two SH2 domains they are almost 10 times more efficient in keeping the phosphatase active.

Insight through the Noonan and LEOPARD syndrome Missense mutations in the PTPN11 gene have been identified as the underlying cause of Noonan syndrome. The majority of altered amino-acid residues are located in or around the interacting surfaces of the N-terminal SH2 domain and the catalytic domain, and are predicted to relieve the intramolecular inhibition caused by binding of the SH2 domain to the catalytic domain (Tartaglia et al., 2002) (Figure 18-13). In some cases there are mutations in residues present in the C-terminal SH2 domain and in the peptide linking the two SH2 domains. However, there is more to the Noonan story because there is no absolute correlation between basal activities of the PTPN11-mutants and the diseases they induce. Some mutations, even those associated with severe symptoms, are without effect on the basal activity or on phosphotyrosine-mediated activation (Keilhack et al., 2005). Mutations in PTPN11 are also linked to the LEOPARD syndrome, so named because of its major clinical features, ECG conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormalities of genitalia, retardation of growth, and deafness (Digilio et al., 2002). Here, a frequently observed mutation occurs in the tyrosine loop, where tyrosine is replaced by a cysteine (Y279C). This is predicted to not only reduce affinity for substrate but it also weakens the inhibitory interaction between the N-SH2 domain and the PTP domain. Consequently, the phosphatase more easily binds to scaffolding adaptors, thus prolonging its open state. Apparently, this by far compensates for the intrinsically reduced phosphatase activity and one may conclude that Y279C too is in effect a gain-of-function mutation (Yu et al., 2013).

Nearly identical clinical features are caused by a gain-of-function mutation of RAF1, which again positions PTP11N in the RAS–MAP-kinase pathway (Pandit et al., 2007). Moreover, mutations that are likely to activate the Ras-guanine nucleotide factor SOS1 can also cause Noonan syndrome (Shannon and Bollag, 2007). In conclusion, both experimental data and genetic analysis of disease reveal that the tyrosine phosphatase PTPN11 acts as an activator of the RAS–MAP-kinase pathway and functions at the level of stimulation of SOS1 (Figure 18-14). How phosphatase activity plays a role in all this however remains elusive.

Noonan is an autosomal dominant disorder characterized by short stature and multiple developmental abnormalities including congenital heart and skeletal malformations (Andersen et al., 2004; Noonan, 1968). The incidence of this syndrome is high—between 1 in 1000 to 2500 live births and in a survey of more than 100 unrelated cases, about 50% displayed a mutation in PTPN11. There is an even higher incidence of mutations among familial cases (Tartaglia et al., 2001). All these are missense mutations that cause a “gain of function.” The clinical features of LEOPARD syndrome are very similar to the Noonan syndrome but the underlying mutations are different.

Gain-of-function mutations in PTPN11 are also linked to some leukemias (Tartaglia et al., 2003). This is perhaps not surprising given its stimulatory role in the Ras-MAP-kinase pathway. Despite some uncertainty with respect to the exact mechanism of cell transformation, PTPN11 mutants provide the first example of a gain-of-function mutation in a PTP that is the underlying cause of a human disease.

Web resource

For more information about Noonan- and LEOPARD syndrome and juvenile myeolomonocytic leukemia, consult the OMIM database, entry numbers 163950, 151100, and 607785, respectively. URL: http://www.omim.org/.

Classical receptor-like PTP phosphatases

PTPRJ, a cell density- enhanced PTP

For PTPRJ (formerly DEP-1), matters are just a bit clearer. This receptor tyrosine phosphatase was originally discovered by probing a HeLa cell cDNA library with a PCR fragment encoding the catalytic domain of a putative PTP. The receptor type PTP isolated is weakly expressed in non-confluent cultures of lung or foreskin fibroblasts, but is highly expressed in dense cultures (Ostman et al., 1994). Later, genome analysis revealed that one of the mouse genes for susceptibility to colorectal cancer encodes a receptor tyrosine phosphatase, PTPRJ. Loss of heterozygosity of the human gene was noted in 19 out of 39 colorectal adenocarcinomas (Ruivenkamp et al., 2002). PTPRJ is the first tyrosine-specific PTP to be assigned a convincing role as a tumor suppressor having relevance to the development of human cancers. Other tyrosine phosphatases have followed and loss-of-function mutations in PTPRD, PTPRT, PTPN12, and PTPN13 are also linked to enhanced cancer incidence (Julien et al., 2011).

PTPRJ acts by dephosphorylating receptor tyrosine kinases, thereby preventing the formation of signaling complexes. It also was shown to dephosphorylate CBL an E3-ubiquitin ligase that plays a role in the endocytosis (and subsequent recycling) of receptor tyrosine kinases. Why this affect receptor signaling is not easily answered. Phosphotyrosine-731 of CBL serves as a docking site for the regulatory subunit (PIK3R1 or p85α) of phosphatidylinositol 3-kinase (PI3-kinase) and phosphorylated CBL may thus too contribute to the formation of signaling complexes. Defective CBL is associated with acute myeloid leukemia due to defective recycling of the FLT3 receptor (Sargin et al., 2007).

For a comprehensive review about the role of phosphatases in cancer we refer to Julien et al. (2011).

PTPRC and the regulation of immune cell function

The PTPRC (formerly CD45) classical protein tyrosine phosphatase, expressed in all nucleated cells of hematopoietic origin, first alerted the attention of immunologists because of its great abundance (5–10% of membrane proteins). It is a transmembrane tyrosine-protein phosphatase having a receptor-like extracellular domain (Tonks et al., 1988a) but as with many other receptor-like PTPs, no specific activating ligand has been identified. Its importance became evident with the discovery that T-lymphocytes lacking this antigen fail to become activated through the TCR, although they respond quite normally to stimulation through the

IL2 receptor (Thomas et al., 1985; Thomas, 1989; Byth et al., 1996) A total absence of surface CD45 causes severe combined immunodeficiency disease (Yoo et al., 2000). Conversely, transgenic mice bearing an activating mutation in CD45 display lymphoproliferation, autoantibody production, and severe nephritis (Majeti et al., 2000). Normally, engagement of the TCR by an antigen-presenting MHC, results in phosphorylation of the three ITAM motifs in the ζ-chains of CD3 (CD247) (which is a part of the TCR complex). This occurs through the intervention of the kinase LCK (lymphocyte cell-specific kinase), bound to either CD4 or CD8 (Figure 18-15, also see Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases," Figure 15-2). The fully phosphorylated ζ-chains then serve as docking sites for the formation of a signaling complex that transmits the signal into the lymphocyte. In cells lacking active PTPRC, LCK remains inactive and the subsequent cascade of tyrosine phosphorylations does not occur.

So where does the phosphatase activity of PTPRC come into all this? Normally, LCK activity is suppressed due to autoinhibition arising from the intramolecular interaction of its own SH2 domain with a phosphotyrosine residue situated in its C-terminus (Pc in Figure 18-15), the kinase adopts a "closed conformation." LCK is activated first through dephosphorylation

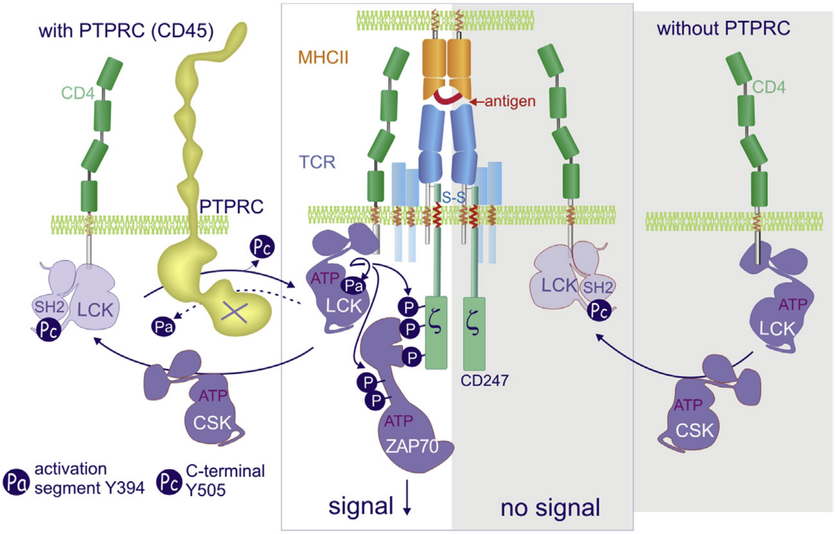


FIGURE 18-15 Role of PTPRC (CD45) in T-cell activation. TCR signaling is dependent on CD45. It maintains LCK in an open (primed) configuration by dephosphorylating the C-terminal tyrosine residue (Pc). Subsequent autophosphorylation in the activation segment (Pa) renders LCK catalytically competent. The kinase CSK does the opposite. It phosphorylates LCK at Pc which makes contact with the SH2 domain, causing it to assume a closed configuration. In the absence of CD45 TCR signaling is prevented.

by PTPRC, which exposes the kinase active site, and then through autophosphorylation in the activation segment (Yamaguchi and Hendrickson, 1996). The fully active LCK phosphorylates the ITAM motifs of the ζ-chains (CD247) but the process only becomes efficient when engagement of the TCR by MHC peptides brings the catalytically competent LCK, bound to CD4 or CD8, into closer juxtaposition with the ζ-chains. From this, it appears that PTPRC maintains a pool of LCK, associated with CD4/CD8, in a primed state. In this way, engagement of the MHC peptide with the TCR leads to LCK-mediated phosphorylation of the TCR ζ-chains and thereby initiates TCR signal transduction (Alexander, 2000).

Important as it may be it is still difficult to understand how PTPRC orchestrates the TCR-response in particular in light of the finding that in T-cells it is also capable of removing the phosphate from the activation segment of LCK (Pa in Figure 18-15). Thus it has the potential to act as an activator and as an inhibitor. What tips the balance is not clear. This duality does not apply for B-cells where PTPRC only removes the C-terminal phosphotyrosine and has an undisputed activator role (Zikherman et al., 2012). Moreover, co-localization of PTPRC and the TCR change with time. Although it is necessary to provide primed LCK at a later stage, when TCR and adhesion-molecule microclusters have formed it is excluded so that it does counteract all the tyrosine phosphorylation events. Only much later is CD45 allowed to return into the clusters so to facilitate termination of the activation process (Figure 18-16).

In addition to LCK, FYN and the Janus kinases (JAK) are also substrates of PTPRC (Irie-Sasaki et al., 2001). The JAK kinases are phosphorylated and activated following cytokine receptor ligation (INFα,

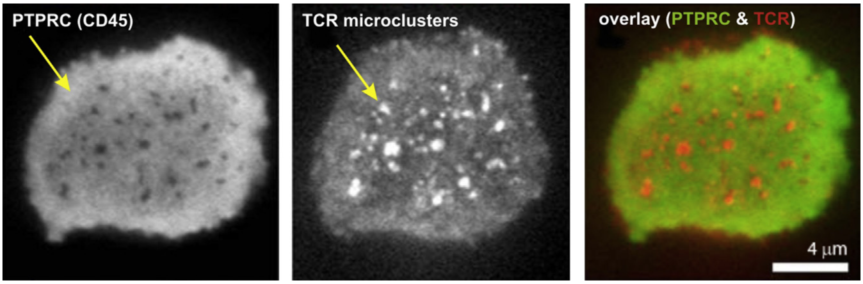


FIGURE 18-16 Localization of PTPRC (CD45) in the regulation of T-cell activation. The fluorescence images are of T-cells that have been placed on a lipid monolayer doped with MHC-II and ICAM-1. They form adhesive structures called microclusters. While CD45 is necessary for the supply of primed LCK (Figure 18-15), it is temporarily excluded from the TCR microclusters. This avoids immediate down-regulation of the early tyrosine phosphorylation-based signaling events. Only later (after ~20 min), when so-called central supramolecular activation clusters are formed (not shown), is CD45 allowed to participate again and to terminate the activation process. *From Varma et al. (2006). Courtesy of Michel Dustin, New York, USA.*

IL3) and in turn phosphorylate the cytoplasmic tail of these receptors to create docking sites for proteins bearing an SH2 domain. Among these are the STAT transcription factors and members of the SRC family of kinases. Tyrosine phosphorylation of STAT causes homo- or heterodimerization which induces translocation to the nucleus. Bone marrow-derived mast cells lacking PTPRC respond more vigorously to IL3 with respect to JAK2 activation, phosphorylation of STAT3 and -5 and proliferation.

Receptor-like tyrosine phosphatases shape the developing synapse

The term receptor-like infers that the extracellular segments are expected to bind ligands. Indeed, three receptor-like tyrosine phosphatases, PTPRF (LAR), PTPRD (RPTPδ), and PTPRS (RPTPσ), were shown to act as adhesion molecules. All three contain fibronectin and Ig-like repeats typical of protein–protein interaction functions. Return to Figure 18-4 for the domain architecture of PTPRF. The three receptor-like tyrosine phosphatases either interact with heparan-sulfate (HS) structures (polysaccharides) in the extracellular matrix or with leucine-rich-repeat (LRR) or Ig domain-containing counter-receptors such as NTRK3 (receptor tyrosine kinase), NGL3, IL1RAP, and members of the SLITRK family (Takahashi and Graig, 2013) (Figure 18-17). In the example of RTPRS, HS and leucine-rich motifs compete for similar binding sites on the Ig-domains, meaning that cell–cell and cell-extracellular matrix contacts compete with each other (Coles et al., 2014).

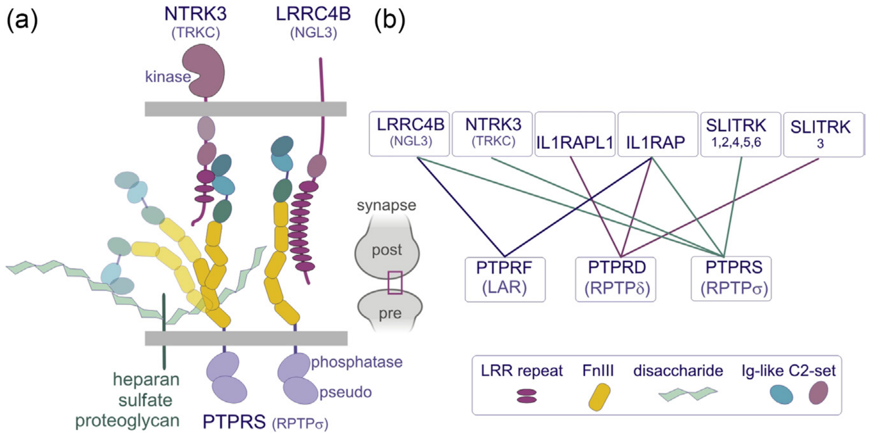


FIGURE 18-17 Receptor-like tyrosine phosphatases as adhesion molecules. (a) The receptor-like protein tyrosine phosphatases PTPRD, PTPRF, and PTPRS act as adhesion molecules and play a role in the maturation of neuronal synapses. They also can bind to heparin-sulfate proteoglycans. (b) Counter-receptors of the receptor-like tyrosine phosphatases are the LRRC4B, NTRK3, IL1RAP, and members of the SLITRK family. *Information from Takahashi and Graig (2013) and Coles et al. (2014).*

For a structural view of LRR, return to Chapter 13, "Activation of the Innate Immune System: The Toll-Like Receptor-4 and Signaling through Ubiquitinylation," Figure 13-2.

These receptor-like tyrosine phosphatases were found to operate in the brain where they have three general functions. One is to mediate synaptic cell–cell adhesion. The second is to mediate presynaptic differentiation, meaning local recruitment of vesicle-docking and vesicle-recycling machinery. The third, indirectly, is to trigger postsynaptic differentiation, involving the recruitment of specific neurotransmitter receptors and their signaling partners. We have discussed the presynaptic membrane and signal propagation briefly in Chapter 8, "Nuclear Receptors," section "Consolidating memories with glucocorticoids." They thus qualify as "synaptogenic" proteins. Whether tyrosine phosphatase activity of the D1 domain is essential for presynaptic differentiation has not been determined (Takahashi and Graig, 2013).

Regulating receptor-like PTP phosphatases

In direct contrast with the kinases, it appears that dimerization may signal "off" for the receptor tyrosine phosphatases. In order to test this idea, cells have been generated that express a chimeric PTPRC (CD45) molecule that comprises its intracellular phosphatase-containing segment coupled to the extracellular domain of the EGF receptor. Application of EGF, expected to dimerize the receptor is then found to switch off the phosphatase activity (Desai et al., 1993). By analogy to RPTPA (Jiang et al., 1999) and PTPRF (LAR), it was proposed that PTPRC might form a symmetrical protein dimer in which the catalytic site of one component is blocked by a wedge structure formed by its partner (Figure 18-18). Separation of the two, exposing the catalytic site, would constitute the activation mechanism. In favor of this idea, the wedge region in PTPRC is conserved at the sequence level and "knock-in" mice carrying a glutamate to arginine mutation in the wedge are subject to enhanced lymphoproliferation.

Against this, however, structural analysis of PTPRC has provided no evidence of dimerization and nor did it reveal the presence of the proposed wedge (Nam et al., 2005). The catalytic site of D1 (membrane proximal catalytic domain) appears to be unobstructed. This study did, however, indicate the importance of the catalytically inactive D2 domain (highly conserved within the tyrosine phosphatase family and across species). It concerns two flexible loop structures, acid and basic. Removal of the acidic loop diminishes CD3-mediated activation of T-cells. Moreover, phosphorylation of serine residues in the acidic loop by casein kinase-2 appears to play a role in PTPRC function. From this we conclude that D2 serves an important purpose, though what this might be is still far from clear.

Recent data from experiments employing analytical ultracentrifugation to examine the catalytic domain oligomerization of a number of receptor-like tyrosine phosphatases revealed densities that correspond to only single domains. The determined molecular masses were in the range of 70 kDa, which is expected for the intracellular segment of a tandem-repeat (cytoplasmic domain of PTPRC (CD45) is 700 amino acids of which 550 comprise the two phosphatase domains). An exception is PTPRG which sediments as a dimer but the dimer does not reveal a wedge-like interaction (Barr et al., 2009).

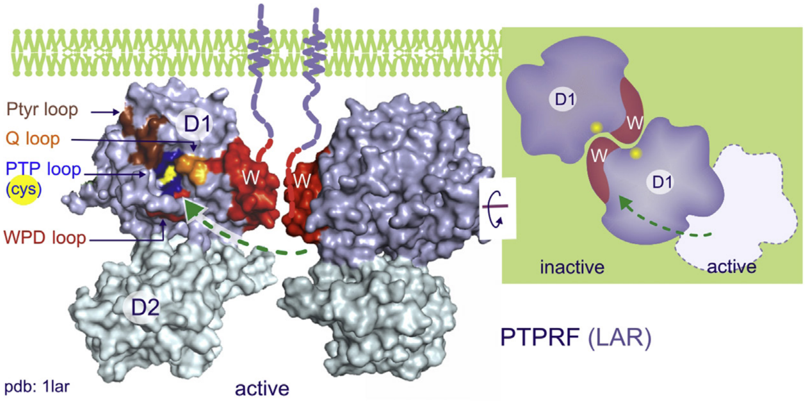


FIGURE 18-18 Inhibition of PTPRF (LAR) through dimerization. Dimerization is the turn-off signal for PTPRF. Inhibition is thought to occur through reciprocal interaction of the two wedge structure (in red) with the catalytic site (blue/yellow) which impedes substrate access. It is unclear whether or not ligands are involved in all this, and if they are, it is unclear how they act to keep the receptors apart. The figure shows the structure of the D1 and D2 domains of PTPRF (LAR). D1 is catalytically active; D2 is a pseudo-phosphatase domain (inactive). Characteristic features of tyrosine phosphatases are indicated (Ptyr loop, Q loop, WPD loop, and the PTP loop,) which harbors the catalytic cysteine residue (in yellow). The sketch shows the organization of the D1 domains in the active (separated) and inactive (closely apposed) states.

Class I DUSP

The DUSP form a subfamily of the tyrosine-protein phosphatases. Under in vitro conditions, these enzymes catalyze the dephosphorylation of all three phosphoamino acids, phosphothreonine, phosphoserine, and phosphotyrosine. Examples include members of the subfamilies such as MAP-kinase phosphatases (DUSP1-16), the CDC14-type phosphatase CDKN3, members of CDC25, or PTEN (return to Figure 18-2). While sharing no overall sequence-similarity between themselves, nor with the tyrosine-specific phosphatases the presence of the conserved PTP signature motif ((I/V)HC-X_5-R) indicates that they are members of this wider family. The catalytic domains of DUSP3 (formerly VHR) and CDKN3 (formerly KAP) possess core structural features similar to the classic PTP proteins but appear to be truncated versions. Importantly, they lack the phosphotyrosine-binding loop and consequently the active site cleft has a depth sufficient to accommodate both phosphotyrosine and phosphothreonine. Fifty-nine DUSP have been characterized which are subdivided into seven groups (Figure 18-19).

Regulation of MAP-kinases by dual-specificity protein phosphatases

Members of the MAP-kinase family are phosphorylated in the TEY sequence by dual-specific kinase MAP2K1 or -2 (MEK1 and -2) (return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Figure 10-27). They are dephosphorylated by the MAP-kinase phosphatases, of which some are PPP phosphatases (PPP2CA and PPP2CB) while others are protein tyrosine phosphatases (PTPRR, PTPN5, and PTPN7) (Keyse, 2000; Arkinstall and Nichols, 2000) (Table 18-1). Yet, others are DUSP. Ascertaining the roles of the specific MAP-kinase phosphatases in vertebrates has been hindered by the great diversity of phosphatase enzymes that inactivate MAP kinases and also their functional redundancy (Farooq and Zhou, 2004). Rapid inactivation of MAPK2 (ERK1) in PC12 cells reflects, in part, dephosphorylation of phosphothreonine (Alessi et al., 1995) whereas in other systems, such as rat-1 fibroblasts, a dominant role for members of the PTP family is evident, leading to dephosphorylation of the tyrosine residue (Pulido et al., 1998).

A remarkable degree of selectivity between substrate and phosphatase is achieved through the interaction of DUSP6 (formerly MKP3) to a common docking domain (CD) adjacent to the catalytic domain on MAPK1 (ERK2) (Figure 18-20). Binding of DUSP6 then causes rearrangement and activation of the catalytic site (Camps et al., 1998). Importantly, catalytic activation of DUSP6 mirrors the selectivity of the docking domain, as the different members belonging to the JNK and p38 groups, are neither able to bind nor to increase its catalytic activity. The docking site on MAPK1 (ERK2) is also its point of interaction with the upstream kinase MAP2K1 (MEK1) and the downstream transcription factor ELK1. Thus, all these components compete for the same binding site (Tanoue et al., 2000). It is not certain whether this mechanism of activation applies to all dual-specificity MAPK phosphatases. For docking sites, return to Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Figure 10-20.

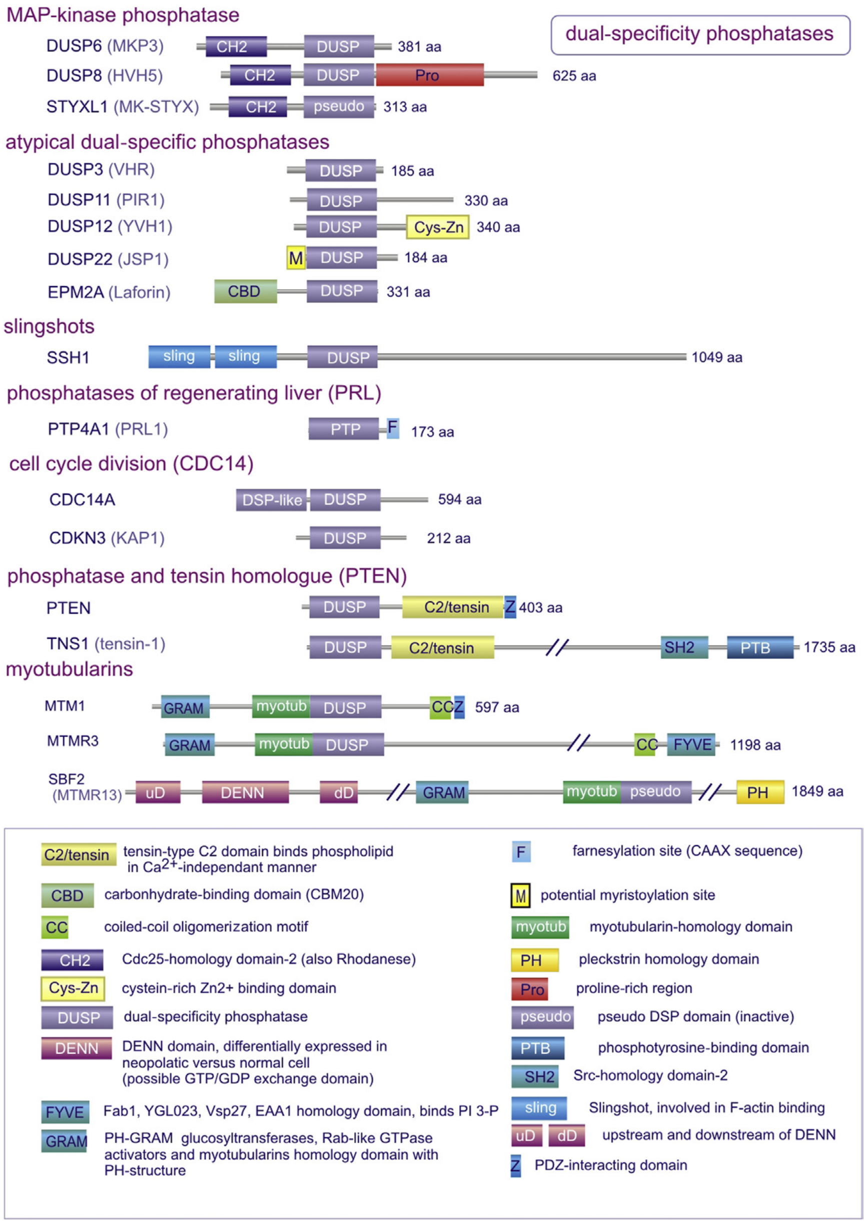

dual-specificity phosphatases
MAP-kinase phosphatase
DUSP6 (MKP3) CH2 DUSP 381 aa
DUSP8 (HVH5) CH2 DUSP Pro 625 aa
STYXL1 (MK-STYX) CH2 pseudo 313 aa
atypical dual-specific phosphatases
DUSP3 (VHR) DUSP 185 aa
DUSP11 (PIR1) DUSP 330 aa
DUSP12 (YVH1) DUSP Cys-Zn 340 aa
DUSP22 (JSP1) M DUSP 184 aa
EPM2A (Laforin) CBD DUSP 331 aa
slingshots
SSH1 sling sling DUSP 1049 aa
phosphatases of regenerating liver (PRL)
PTP4A1 (PRL1) PTP F 173 aa
cell cycle division (CDC14)
CDC14A DSP-like DUSP 594 aa
CDKN3 (KAP1) DUSP 212 aa
phosphatase and tensin homologue (PTEN)
PTEN DUSP C2/tensin Z 403 aa
TNS1 (tensin-1) DUSP C2/tensin SH2 PTB 1735 aa
myotubularins
MTM1 GRAM myotub DUSP CC Z 597 aa
MTMR3 GRAM myotub DUSP CC FYVE 1198 aa
SBF2 (MTMR13) uD DENN dD GRAM myotub pseudo PH 1849 aa
C2/tensin tensin-type C2 domain binds phospholipid in Ca2+-independant manner
CBD carbonhydrate-binding domain (CBM20)
CC coiled-coil oligomerization motif
CH2 Cdc25-homology domain-2 (also Rhodanese)
Cys-Zn cystein-rich Zn2+ binding domain
DUSP dual-specificity phosphatase
DENN DENN domain, differentially expressed in neopolatic versus normal cell (possible GTP/GDP exchange domain)
FYVE Fab1, YGL023, Vsp27, EAA1 homology domain, binds PI 3-P
GRAM PH-GRAM glucosyltransferases, Rab-like GTPase activators and myotubularins homology domain with PH-structure
F farnesylation site (CAAX sequence)
M potential myristoylation site
myotub myotubularin-homology domain
PH pleckstrin homology domain
Pro proline-rich region
pseudo pseudo DSP domain (inactive)
PTB phosphotyrosine-binding domain
SH2 Src-homology domain-2
sling Slingshot, involved in F-actin binding
uD dD upstream and downstream of DENN
Z PDZ-interacting domain

FIGURE 18-19 Domain architecture of some of the dual-specificity phosphatases. Although characterized by the presence of a common signature motif, the dual-specificity phosphatases are more diverse than the classic PTP proteins. As a consequence numerous small subfamilies exist that possess a truncated version of the catalytic domain. Among other differences, this results in a shallower catalytic pocket. Both catalytically competent and pseudo-phosphatases have been identified. Some accessory domains as well as interaction sites or posttranslational modification sites are highlighted. Gene names from HUGO gene-nomenclature database. *Information from* Tonks (2006).

TABLE 18-1 MAP-kinase phosphatases

Dual-specificity phosphatases (DUSP)	
DUSP1 (MKP-1)	- Nuclear, targets MAPK1, -3 (ERK2, -1), and MAPK14 (p38a)
DUSP2 (PAC1)	- Growth factor or stress-induced, targets MAPK1, -3
DUSP4 (MKP-2)	- Expressed among immediate early genes, targets MAPK1, -3
DUSP5 (HVH3)	- Growth factor or stress induced, targets mainly MAPK3
DUSP6 (MKP-3)	- Cytosolic, targets mainly MAPK1, -2, -7?
DUSP7 (MKP-X)	- Targets MAPK1, -14
DUSP9 (MKP-4)	- Targets MAPK14
DUSP10 (MKP-5)	- Targets MAPK11–14 (p38)
Classical tyrosine phosphatases (PTPR, PTPN)	
PTPRR (PCPTP1)	- Neuronal
PTPN5 (STEP)	- Targets MAPK1, -3 (ERK), and MAPK11-14 (p38)
PTPN7 (HePTP)	- Lymphoid, MAPK1, -3 (ERK), and MAPK11-14 (p38)
Serine/threonine phosphatases (PPP)	
PPP2CB	- Targets All MAP-kinases
PPP2CA	- Targets MAPK11–14 (p38) and MAPK8–10 (JNK)

Three categories of MAP-kinase phosphatases are recognized. The first comprise dual-specificity phosphatases. Some members are directly induced by MAP kinases (immediate early genes) and are localized mainly in the nucleus whereas others are not induced by MAP kinases and act in the cytoplasm. The second category comprises members of the tyrosine phosphatase family (PTP) and the third comprises the serine/threonine phosphatases PPP2CA and PPP2CB. Not all members of the MAP-kinase family are equally susceptible as substrates, preferences are indicated for some.

Physiological role of the dual-specificity MAPK phosphatases

The archetypal DUSP is the VH1 gene of vaccinia virus (Guan et al., 1991). A mammalian homologue of VH1 was discovered as a product of a growth factor-induced early response gene (Charles et al., 1992; Sun et al., 1993). Biochemical analysis revealed it to be an MAP-kinase phosphatase (DUSP1, formerly MKP1).

Addition of serum to quiescent cells induces rapid but transient phosphorylation and activation of MAPK1 (ERK2). This is sufficient to induce gene transcription and to promote entry into the G1 phase of the cell cycle (Assoian, 1997). The activity of DUSP1 is expressed within 20 min and coincides with the dephosphorylation of the pTEpY motif in the activation segment followed by the deactivation of the kinase (see Figure 18-21(a) and see Chapter 10, "Regulation of Cell Proliferation

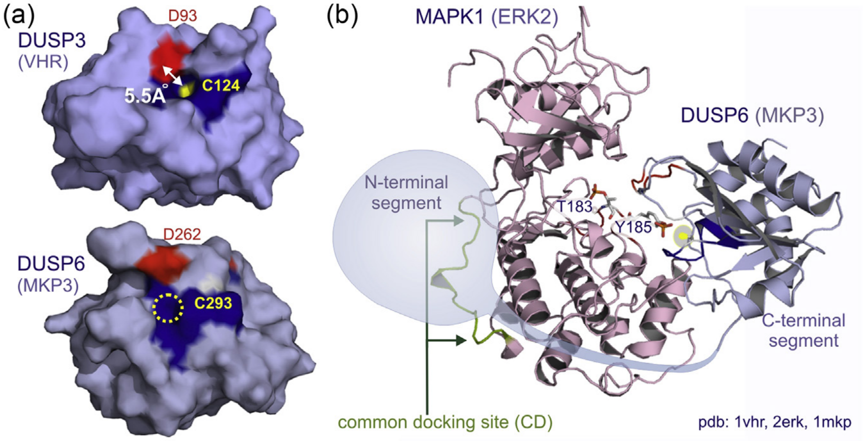


FIGURE 18-20 Structure of the dual-specificity phosphatases DUSP3 and DUSP6. (a) Surface representation of DUSP3 (VHR) and DUSP6 (MKP3). The PTP loop is represented in blue and the aspartates D93 and D262, important for the dephosphorylation reaction, are colored in red. The catalytic pocket measures 5.5 Å. Note that the catalytic cysteine of DUSP6 (C293) is not exposed to the surface (no yellow spot visible). It appears that the binding of MAPK1 alters the active site of DUSP6 so as to expose the catalytic cysteine. (b) Model illustrating the interaction of MAPK1 (ERK2) with DUSP6. The N-terminal segment of the phosphatase binds to the common docking site (CD) at the "back" of MAPK1 whereas the C-terminal segment binds to Y185 in the activation segment. The two segments are connected by a hinge region that bends around the C-lobe of the kinase.

by Receptor Tyrosine Protein Kinases", Figure 10-15). If synthesis of DUSP1 is prevented, or if its catalytic activity is suppressed by mutation, the duration of the activated state of MAPK1 is greatly extended. Conversely, expression of a constitutively activated form of DUSP1 blocks G1-specific transcription and entry into S-phase (Brondello et al., 1995; Sun et al., 1994). Thus DUSP1 acts as a negative regulator of MAPK1, serving to attenuate the growth-factor signal (Figure 18–21(b)). In addition, DUSP1 is a substrate of MAPK1 and when phosphorylated, it becomes less sensitive to ubiquitin-directed proteolysis and as a result, it accumulates (Brondello et al., 1999). Despite all these promising results, this is certainly not the only pathway by which MAPK1 is controlled because none of the mutations in DUSP1 predispose to tumor development. If this pathway was absolutely essential, then one might expect that a tumorigenic mutation would have turned up by now. Moreover, DUSP1-deficient mice appear to develop normally. Their fibroblasts respond normally with respect to the extent and timing of the expression of FOS, indicating that the control of MAP kinase is unperturbed (Dorfman et al., 1996).

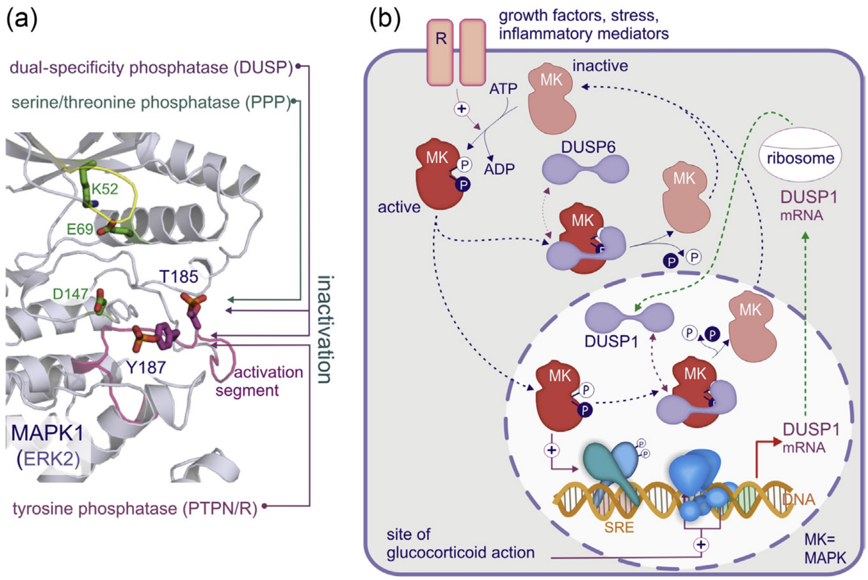

(a)
dual-specificity phosphatase (DUSP)
serine/threonine phosphatase (PPP)
K52
E69
T185
D147
Y187
activation segment
MAPK1 (ERK2)
inactivation
tyrosine phosphatase (PTPN/R)
(b)
R
growth factors, stress, inflammatory mediators
inactive
MK
ATP
ADP
+
DUSP6
active
P
ribosome
DUSP1 mRNA
DUSP1
SRE
DNA
site of glucocorticoid action
MK= MAPK

FIGURE 18-21 Phosphatases and the inactivation of MAP kinases. (a) All the phosphatases cause inactivation through dephosphorylation of one or both of the phosphoamino-acid residues in the activation segment. (b) Another example of phosphatases acting as a reset button. The scheme shows how dual-specificity phosphatases operate in receptor signaling. Receptors activate the MAP-kinase pathway, resulting in the activation of MAPK (members of the ERK, JNK, or p38 subfamilies). A fraction of these operate in the cytoplasm until they are dephosphorylated and inactivated by DUSP6. Others transfer to the nucleus to induce gene expression through phosphorylation of transcription factors. The product of one of the immediate early genes, DUSP1, enters the nucleus where it inactivates MAPK. DUSP1 can also be induced by the synthetic glucocorticoid dexamethasone. Dephosphorylated and inactivated MAPK accumulates in the cytoplasm where it is "recharged" by occupied cell-surface receptors. *Information from Camps et al. (2000).*

We now know that DUSP1 is more active against members of the p38 and JNK kinases (see Table 18-1) (Farooq and Zhou, 2004) and that the ERK members can also be dephosphorylated by DUSP6 (Groom et al., 2006).

DUSP6 plays an important role in the regulation of MAPK in T-lymphocytes. As we age, the immune system becomes less efficient at protecting us from infection. An important contributing factor is an increase in the activation threshold of naive T-cells. This correlates with an age-associated loss of expression of microRNA-181a (miR-181a). When the TCR or naive CD4+ lymphocytes from people of 70 years of age or older are triggered with a peptide antigen, the initial signaling steps, activation of the non-receptor tyrosine kinase ZAP70, and of phospholipase C-γ1, seem unperturbed; yet the cells do not easily respond with clonal expansion. What lacks is the contribution of MAPK1 (ERK2) (Altan-Bonnet and Germain, 2005) and the reason for this is an elevated expression of DUSP6 in naïve T-cells (not in memory T-cells). This is not due to elevated transcription levels but the consequence of a reduced level of expression of miR-181a which normally prevents translation of the mRNA of a number of genes, among which is DUSP6. When expression of DUSP6 is blocked through reconstitution of miR-181a, the T-cells bounce back with a strong response to novel antigens (Li et al., 2007, 2012).

Web resources

- To learn about miRNA, consult the miRBase database run by the University of Manchester, UK (Kozomara and Griffiths-Jones, 2014). URL: www.mirbase.org.
- Targets of miRNA can be found at the target-scan (human) database run by the Bioinformatics and Research Computing group at the Whitehead Institute for Biomedical Research, Cambridge, USA. URL: www.targetscan.org (search for hsa-mir-181a).

DUSP in development

Most investigations of the dual-specificity MAPK phosphatases have focused on their roles in development. A particularly informative experiment, providing a direct demonstration of a physiological role for a DUSP in the regulation of MAP-kinase activity came from studies of dorsal closure during embryogenesis in *Drosophila*. In this process, lateral epithelial cells undergo cytoskeletal changes which enable them to spread out, providing a cover for the dorsal region of the embryo (Sluss et al., 1996) (Figure 18-22(b)). The *Drosophila* homologue of the mammalian JNK, Bsk, induces the expression of *puc*, which encodes a DUSP and negatively regulates activity of Bsk. The activity of Puc must be regulated precisely. Lack of Puc results in a disordered border between the dorsal epithelial cells (“puckered” phenotype) (Figure 18-22(a)). In excess it induces a phenotype characterized by patches of uncovered dorsal openings (Martin-Blanco et al., 1998).

The ERK-specific DUSP6 (MPK3) plays a similar role in the regulation of chicken limb outgrowth. This is initiated by FGF8 released from the epithelial cells that form the apical epidermal ridge. FGF8 causes proliferation of the underlying mesenchymal cells to form buds that extend into the limbs with differentiation of the mesenchymal cells into cartilage and then bone. DUSP6 is one of the genes up-regulated in mesenchymal cells by FGF8. Its inhibition of expression by siRNA, results in cell death in the mesenchyme (Kawakami et al., 2003). In this system, DUSP6 expression is regulated by the PI3-kinase/AKT pathway.

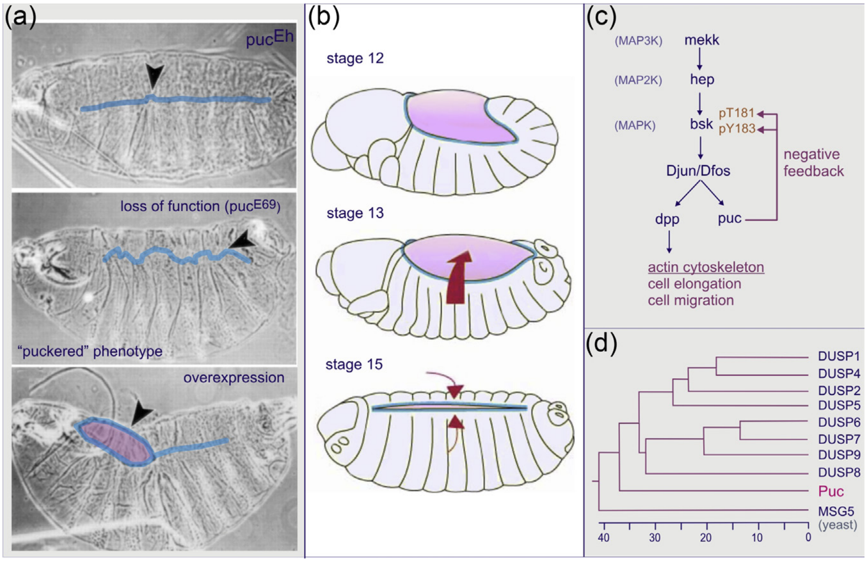


FIGURE 18-22 Dorsal closure in *Drosophila* and the role of the dual-specificity phosphatase *puc*. (a) Phenotypes of *Drosophila* embryos arising as a consequence of altered or enhanced expression of the *puc* gene. The weak mutant *puc*$^{\text{Eh}}$ yields an almost perfect seal between the two sides of the ectoderm. Loss of function (*puc*$^{\text{E69}}$) causes a puckered phenotype, in which the cells of the two borders penetrate each other's territory. Overexpression prevents the leading edges reaching each other and the embryo reveals dorsal openings. *Image modified from Farooq and Zhou (2004). Courtesy of Martinez-Arias, Cambridge, UK.* (b) Stages in dorsal closure during *Drosophila* embryogenesis. The embryos are shown dorsal side up and the anterior (future head region) to the left. At stage 12, a large part of the embryo is still covered by amnioserosa (pink). At stage 13, the ectoderm cell sheet extends toward the dorsal midline (red arrow). This movement takes about 2h and is the consequence of the progressive flattening of cells which extend a leading edge toward the midline. At stage 15, the leading edges of both sides converge. The process of flattening and migration ceases. (c) The signal transduction pathway that controls dorsal closure. Positive control of flattening and migration occurs through induction of dpp (decapentaplegic, member of the TGFβ family of growth factors) by a pathway involving *mekk* (M1K), *hep* (M2K), and *bsk* (M3K). When cells make contact negative feedback due to Puc-mediated dephosphorylation and inactivation of Bsk arrests flattening and migration. (d) Phylogenetic tree analysis of *puc*, yeast MSG5 and human dual-specificity phosphatases. *Image courtesy of Dr Martinez-Arias, Cambridge, UK. Embryonic stages according to Campos-Ortega and Hartsenstein (1984).*

PTEN, a DUSP for phosphatidyl inositol lipids

As indicated at the start of this chapter, interest in protein tyrosine phosphatases was sparked off in the hope they might act as tumor suppressors. However, of the many phosphatases investigated, only very few seem to exert such a role; PTEN is one of them. It provides the best example of gene coding for a PTP in which loss of function is strongly linked with tumor development (Li and Sun, 1997; Li et al., 1997; Cantley and Neel, 1999). Somatic mutations, resulting in its inactivation, occur in multiple sporadic cancer types. Germline mutations are linked to inherited hamartoma and Cowden syndrome (Chapter 16, “Signaling through the Insulin Receptor,” section “PI 3-kinase, regulator of cell size, proliferation and transformation”). Genetic studies in mice too have unambiguously demonstrated that PTEN is a potent tumor suppressor in almost all organs (Knobbe et al., 2008). Thus it came as some surprise to find that it does so, not by controlling the level of tyrosine phosphorylation but through its action as a phosphatase for PI-3,4,5-P3 (Maehama and Dixon, 1998). Protein substrates are not totally excluded. Among the substrates are PTK2 (formerly FAK), the adaptor protein SHC, and possibly the enzyme itself (auto dephosphorylation) (Zhang et al., 2012). However, until today, the major tumor-suppressive function of PTEN can be ascribed to its lipid phosphatase activity (Myers et al., 1998).

The amino-acid sequence of PTEN contains a tyrosine phosphatase signature motif and it displays the structural features of the tyrosine-protein phosphatases (Figure 18-23(a)). In spite of this, PTEN is generally a poor protein phosphatase. It catalyzes the removal of the phosphate from the 3- position of the phosphatidyl inositol PI-3,4,5-P3 (PIP_3) with far greater alacrity (Maehama and Dixon, 1998). Compared with the dedicated protein phosphatases, PTEN possesses positively charged amino acids (CKAGKR) in the PTP loop that may explain its ability to target PIP_3 (Lee et al., 1999). The protein possesses a phosphatase and C2 domain (lipid binding) which associate across an extensive interface, suggesting that the C2 may serve to locate the catalytic domain at appropriate sites on the membrane (Figure 18–23(b)). The C-tail is disordered and carries numerous phosphorylation sites plus a four amino-acid motif that interacts with PDZ domains (protein–protein interaction domain). Phosphorylation of the C-tail not only prevents membrane binding (inhibition of enzyme activity) but it also affects protein half-life (discussed later) (Vazquez et al., 2000).

With its preferred substrate PIP_3, PTEN is positioned at the head of two well-characterized signaling pathways, the one determining cell survival (signals deriving from focal adhesion complexes), the other leading to cell proliferation (signals from receptor protein tyrosine kinases) (return to Chapter 11, “Signal Transduction to and from Adhesion Molecules,” Figure 11-17). In essence, PTEN serves to maintain PIP_3 at a low level. Unlike other players in the PI 3-kinase pathway, there is a lack of functional redundancy for PTEN and this may be the underlying reason for its prominence as a tumor suppressor. As a result of PTEN activity, the recruitment and activation of PH domain-containing enzymes such as AKT and PDK1 are held in check (Alessi et al., 1998). (Loss-of-function mutations that frequently arise in cancers are shown in Figure 18-23(c).)

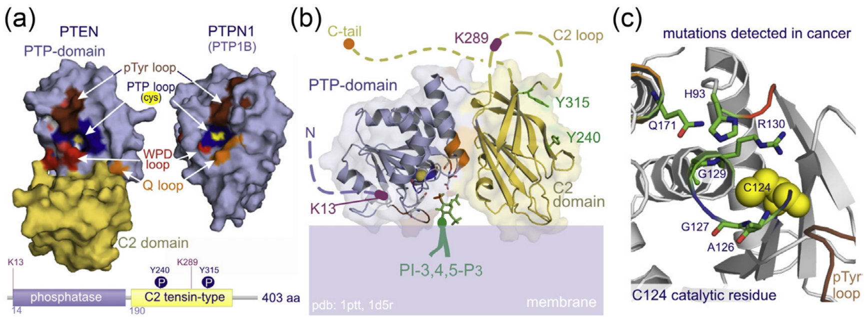


FIGURE 18–23 PTEN, a tyrosine phosphatase look-a-like. (a) Surface representations of PTEN and PTPN1 reveal the characteristic features of the tyrosine phosphatases, with a PTP loop, containing the catalytic cysteine residue (yellow) and the phoshotyrosine, WPD, and Q loops. PTEN contains an additional C2 domain. (b) Cartoon representation of PTEN attached to a phospholipid bilayer. The PIP_3 is projected into the catalytic pocket, close to the catalytic cysteine. The C2 domain (yellow) facilitates binding to the membrane. A number of features which are dealt with within this section are highlighted. The C2 domain has two tyrosine residues, Y240 and Y315, which are phosphorylated by SRC. The C2 domain also contains an unstructured loop of about 23 residues (hatched yellow line) harboring lysine K289, which is ubiquitylated by NEDD4. Another lysine, K13, at the C-terminus is also ubiquitylated by this E3-ligase. Finally the C-terminal tail of PTEN is unstructured (indicated by hatched yellow line with orange dot). (c) Loss of function mutations of PTEN are detected in cancer. The residues frequently mutated (H93, Q171, R130, G127, and A126) all surround the catalytic C124 residue.

Regulation of activity: the open (active) and closed (inactive) state of PTEN

The C2 domain of PTEN binds phosphatidylserine, which is abundant in the inner leaflet of mammalian plasma membranes. In addition, a short N-terminal module selectively binds PI(4,5)P2, which initiates a second conformational change that activates the PTEN phosphatase domain (Campbell et al., 2003). Finally, there is a C-terminus sequence that interacts with PDZ domain-containing proteins. Together they position the enzyme at the membrane and cause enzymatic activity. Given these multiple PTEN-membrane contact points, it was surprising that most of PTEN is in fact cytoplasmic (Vazquez et al., 2006). The 50 amino acid-long C-tail plays an essential role in all this. When it is phosphorylated, in particular at residues S380, T382, T383, and S385, the protein adopts a closed conformation. The tail forms intramolecular bonds with the catalytic site, the C2 domain and even the short N-terminal stretch (Figure 18-24) (Radhar et al., 2009). Casein kinase-2 is responsible for phosphorylation of the above mentioned serine and threonine residues. How phosphorylation and dephosphorylation is controlled remains an enigma. As mentioned previously, PTEN may act as its own phosphatase.

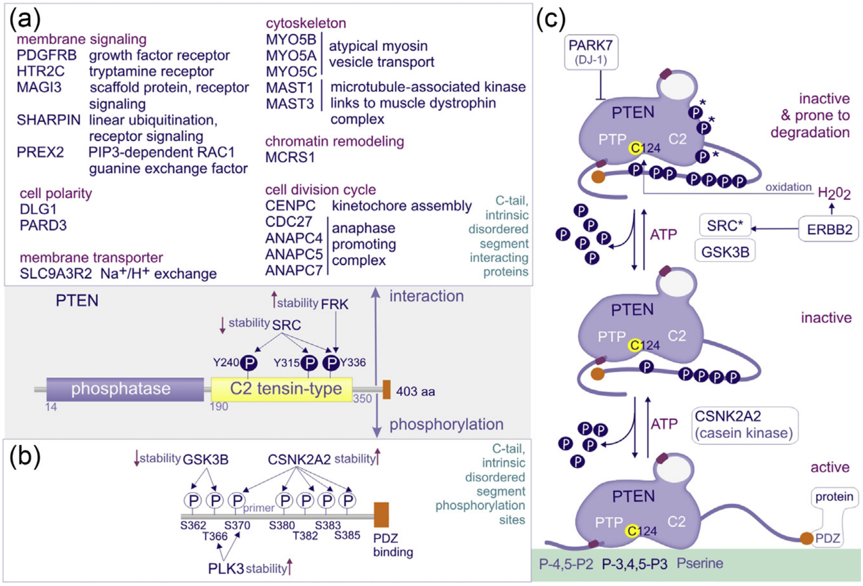


FIGURE 18-24 PTEN a hub for kinases, phosphatases, and protein interactions. (a) The intrinsic disordered C-tail of PTEN serves as a binding site for a large number of proteins. They are involved in membrane signaling, cell polarity, membrane transport, chromatin remodeling, cell division, or they are components of the cytoskeleton. PTEN somehow plays a role in all these functions. (b) The intrinsic disordered C-tail also harbors at least seven phosphorylation sites for three different protein kinases. These determine protein stability and play a role in the activation state. (c) Fully phosphorylated PTEN is inactive, cannot access the membrane, and is prone to degradation. Removal of the SRC phosphotyrosines and GSK3B phosphoserines enhances stability but still prevents access to the membrane. The C-tail makes contact with the C2 domain, the catalytic site wand with the N-terminal sequence. Loss of casein kinase phosphoserines and threonines causes membrane binding. Four contact sites have been revealed: the N-tail binds P-4,5-P2, the catalytic site binds the substrate P-3,4,5-P3, the C2 domain interacts with phosphatidylserine, and the C-terminal amino acids (orange dot) bind PDZ domain-containing proteins.

Redox regulation

Another mechanism that can potentially regulate the catalytic activity of PTEN is direct oxidation by ROS. H_2O_2 can oxidize Cys-124 in the active site (for details see Figure 18-10) (Lee et al., 2002). Oxidative inactivation of PTEN has been reported in studies describing the use of hydrogen peroxide or endogenous ROS production in macrophages and it may play a role in transiently boosting insulin signaling. PTEN activity can also be indirectly inhibited by oxidation through modulation of PTEN-binding partners. Oxidation of a cysteine of PARK7 (also known as DJ-1) leads to binding to PTEN and subsequent inhibition of the PTEN lipid phosphatase activity (Kim et al., 2005, 2009) (Figure 18-24). PARK7 emerged from a screen of PTEN signaling in *Drosophila* (Kim et al., 2005).

It owns its gene name to the observation that defects in the protein have also been noted in autosomal recessive early-onset Parkinson's disease.

PARK7, protein name DJ-1 (named after two students, Daisuke Nagakubo and Junko Maeda), was originally cloned as a putative oncogene capable of transforming NIH-3T3 fibroblasts in cooperation with RAS (Nagakubo et al., 1997). Several lines of evidence indicate that PARK7 plays a role in human tumorigenesis: breast cancer patients have elevated levels of circulating PARK7, cellular protein levels are increased in primary nonsmall cell lung carcinoma samples, and its expression is decreased in human lung cancer cells treated with the chemotherapy drugs paclitaxel (metaphase inhibitor) and U0216 (MAP2K (MEK) inhibitor).

Regulation of PTEN protein stability

Protein half-life also plays a role in the control of PTEN activity. Worth mentioning is the regulation by SRC which reduces the half-life of the enzyme and it is possible that this may be involved in SRC-mediated cell transformation (Figure 18-24). Its relevance was demonstrated by the observation that Herceptin, a humanized monoclonal antibody that targets the ERBB2 receptor (EGF receptor) and which can suppress some malignant breast tumors, reduces SRC-mediated PTEN phosphorylation so enhancing its half-life (Nagata et al., 2004). Likewise, phosphorylation of the C-tail by GSK3B, which only takes place if the S370-primer site is phosphorylated by casein-kinase-2, also decreases protein stability and has an overall inhibitory effect on PTEN (Figure 18-24, panel (b)).

Nuclear Action of PTEN

With respect to the suppression of cell transformation, the action of PTEN at the cell membrane is perhaps only half the story. Recent studies have demonstrated a role for PTEN in the nucleus (Figure 18-25). Nuclear translocation requires ubiquitylation in a process driven by NEDD4, a HECT-type E3-ubiquitin ligase (return to Chapter 13, "Activation of the Innate Immune System, The Toll-Like Receptor and Signaling through Ubiquitinylation," essay "Ubiquitinylation and sumoylation"). Mono-ubiquitinylated PTEN is recognized by the nuclear import machinery (comprising importin and RanGTP) and enters the nucleus (Trotman et al., 2007) where it affects expression of RAD51, involved in the prevention of DNA double-strand breaks. Also it associates directly with CENPC, an integral component of the kinetochore, and helps to stabilize the centromere, needed for accurate segregation of the chromosomes during mitosis. Together these actions are said to safeguard chromosome integrity and thus prevent genetic anomalies (Shen et al., 2007). Whether or not PTEN also regulates the activity of AKT in the nucleus

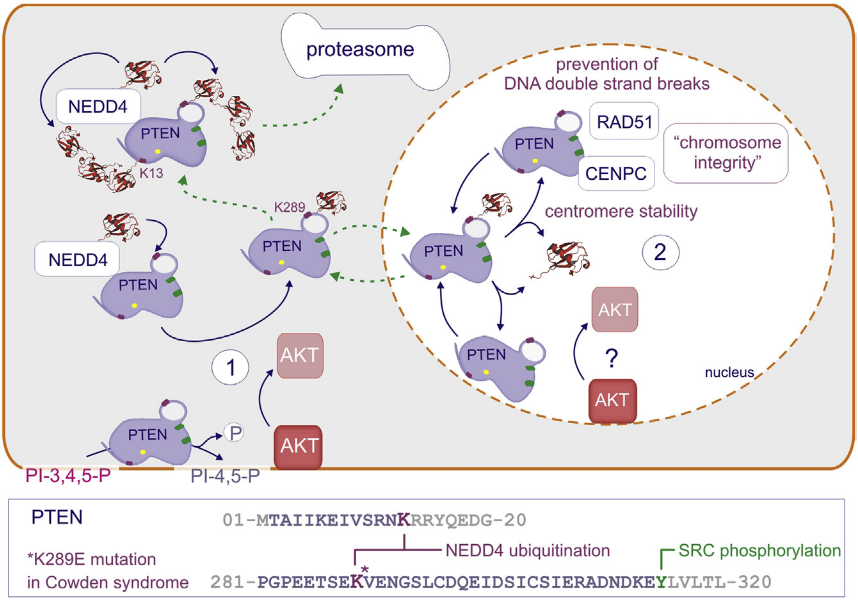


FIGURE 18-25 PTEN: action at two locations. In the cytoplasm, PTEN holds the PI3-kinase pathway in check, thereby regulating the activity of AKT (1). Following mono-ubiquitylation by the HECT-type E3-ligase NEDD4, PTEN enters the nucleus and then after de-ubiquitylation it acts to up-regulate the expression of RAD51, a protein that prevents double-strand breaks of DNA. It also associates with CENPC, an integral component of the kinetochore, and together they assure centromeric stability (needed for accurate segregation of the chromosomes during mitosis). Both actions ensure chromosome integrity and prevent genetic anomalies that eventually may lead to cell transformation. It is not clear if PTEN affects the activation state of AKT in the nucleus. Patients with Cowden syndrome lack the K289 ubiquitinylation site and as a consequence they have a reduced nuclear action of PTEN. This may explain, in part, the formation of tumor-like growths (hamartomas) and the increased propensity for the development of certain forms of cancer.

is not yet clear. The importance of all this has been highlighted by the finding that lysine to glutamate mutation (K289E) occurs in some cases of Cowden syndrome and as a consequence PTEN fails to enter the nucleus. Not only this, but failure of nuclear localization also renders PTEN more vulnerable to poly-ubiquitylation, resulting in its recognition by the proteasome and leading to its destruction (Figure 18-25). Finally, we note that PTEN can inhibit cell-cycle progression by modulating the activity of the anaphase-promoting complex/cyclosome (APC/C) in the nucleus in a manner that is independent of its enzymatic activity (see below "in cauda venenum") (Song et al., 2011).

In Cauda Venenum

PTEN has a highly disordered, functionally versatile, C-tail encompassing amino acids 351–403. It includes the short motif that binds PDZ domain-containing proteins. Whereas structured proteins adopt energetically stable three-dimensional conformations with minimum free energy intrinsically disordered proteins (or protein segments), due to their unique amino-acid sequence arrangements, cannot adopt energetically favorable conformations and lack stable tertiary structure. This structural plasticity allows intrinsic disordered segments to operate within numerous functional pathways, conferring multiple regulatory functions. The functional relevance is shown indirectly by the observation that the C-tail harbors very few mutations (or natural variants) whereas the rest of the protein carries roughly eight-times more per 50 amino-acid stretches (Malaney et al., 2013). This can be taken to mean that changes in the tail are immediately deleterious for the organism. The PTEN primary interactome reveals some 40 proteins which bind to known regions of PTEN and of these 24 bind the disordered tail. Among them are the already mentioned CENPC and components of the APC, such as APC3, 4, 5, and 7. In total some 340 interactions have been revealed but the protein regions concerned are not yet revealed. Of the PTEN-interacting proteins, approximately 80% carries intrinsically disordered segments. The impact of these interacts is still a subject of research.

PROTEIN SERINE/THREONINE PHOSPHATASES

The serine/threonine phosphatases are oligomers and characterized by their association with targeting subunits. These direct them to particular locations, so restricting their action to a limited range of substrates, and they contribute to the docking of substrate. The first serine/threonine phosphatase was recognized in 1945 by Gerty and Carl Cori and initially named PR, for prosthetic group removing enzyme (prosthetic groups such as FAD, NAD, FMN were thought to be required for enzymes involved in metabolic activity). It inactivates the active version of glycogen phosphorylase (glycogen phosphorylase *a*). PR turned out to be protein phosphatase-1 (PP1), a complex comprising a catalytic (PPP1CA) and varying regulatory subunits (PPP1R3A, 3B, and others). It dephosphorylates the phosphoserine-14 and this, through a conformational change, renders the enzyme less active and more susceptible to inhibition by glucose-6-phosphatase ("end product") and by ATP (Cori and Cori, 1945; Stralfors et al., 1985). For more information return to Chapter 2,"An Introduction to Signal Transduction," section,"Putting the components together: the adrenaline to glycogen phosphorylase-signaling pathway." Related serine/threonine phosphatases have since been identified and they make up the subfamily of PPP phosphatases (return to Figure 18-2). Other serine/threonine phosphatases are the Mg^{2+}/Mn^{2+}-dependent phosphatases (PPM) (Cohen, 1997, 1994) and the aspartate-based protein phosphatases (CTD). We elaborate on some of the members of the PPP subfamily and ignore more or less the existence of the other two subfamilies. The PPP family is characterized by the presence of three invariant amino-acid motifs in the catalytic domain (Figure 18-28) and two metal ions required for their activity, though their identity remains controversial, possibly $Mn^{2+} + Mn^{2+}$ or $Mn^{2+} + Fe^{2+}$.

Because they are relatively nonspecific in their action, it was thought that the small number of PPP enzymes would be sufficient to balance the effects of the numerous kinases (in a rather nonselective fashion) (Cohen, 1989). Some functional diversity then became apparent with the discovery of inhibitors that affect a restricted range of phosphatases. As examples, ciclosporin A, widely used as an immunosuppressive agent after organ transplantation, is a selective inhibitor of PPP3CA (calcineurin) (return to Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases," Figures 15-5 and 15-6). Okadaic acid, a tumor promoter, is a potent inhibitor of PPP1CA/B/C and PPP2CA/B. Real diversity came to light when it was realized that the purified activities represent only the catalytic subunits, but that in the cellular environment, they are coupled with targeting or regulatory subunits. The subcellular distributions, substrate selectivities, and catalytic activities are largely determined by these regulatory subunits. For example, PPP1CA is active against a wide range of peptide and protein substrates and so its activity must be carefully limited. Under normal conditions in muscle, it is coupled with PPP1R3A (formerly Gm) which has very high affinity for glycogen ($K_{app} \sim 6\,nM$) (Figure 18-26). This ensures that its free form is kept at vanishingly low levels. In the situation of severe glycogen depletion or upon detachment due to phosphorylation of PPP1R3A, another regulatory subunit PPP1R1A, also known as inhibitor-1, sequesters the catalytic subunit thus ensuring low-intracellular concentrations. In these ways, PPP1C1A is prevented from acting as a loose cannon, randomly dephosphorylating any phosphoprotein that might come in range. The other enzymes of glycogen metabolism, phosphorylase-kinase, phosphorylase,

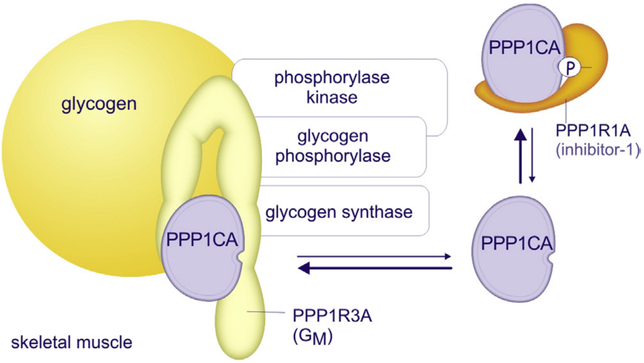


FIGURE 18-26 Serine/threonine phosphatases targeting subunits and inhibitor proteins. PPP1CA, involved in glycogen metabolism, never exists in a free soluble state. It is bound either to its regulator subunit PPP1R3A (Gm), associated with glycogen (forming the enzyme complex "PP1G") or it is bound to the phosphorylated inhibitory subunit PPP1R1A (inhibitor-1), which prevents uncontrolled de-phosphorylation of other substrates.

and glycogen synthase are also tightly bound to glycogen and all three are good substrates for dephosphorylation by PPP1CA/PPP1R3A (PP1/Gm). The domain architectures of some representative members of the PPP phosphatases and their regulatory subunits are depicted in Figure 18-27. For a description of the structure, conserved residues and the catalytic mechanism we refer to Figure 18-28.

Regulation of PPP phosphatases

Phosphorylation of the catalytic subunits

The C-terminal region of the catalytic domains is critical for the communication of regulatory signals to the active site. For instance, phosphorylation by CDK2 of a C-terminal threonine residue (T320) in PPP1CA inhibits the activity in a manner dependent on the point in the cell cycle. This prevents the reversal of CyclinE/CDK2-mediated phosphorylation of the retinoblastoma protein (RB1) and therefore facilitates cell-cycle progression toward the S-phase (Ishii et al., 1994; Dohadwala et al., 1994) (Figure 18-29).

Regulation by intramolecular domain interaction

The widely expressed PPPC5 participates in several stress-activated cellular-signaling pathways involving the kinases subfamilies p38 and JNK. It also binds to the heterotrimeric G-protein subunits Gα12/13 which gives the clue that it may be involved in responses to 7TM receptors (Yamaguchi et al., 2002).

The in vitro basal activity of PPP5C is extremely low. Moreover, whereas PPP1C, PPP2C, and PPP4C exist as dimers or trimers, with the catalytic subunit bound to a regulatory subunit (next section), PPP5C is a monomer and regulation is due to the presence of a tetratricopeptide repeat (TPR, 34 residues) domain present at the N-terminus. The TPR-domain blocks substrate access to the catalytic cleft and this inhibited conformation is stabilized by the αJ-helical domain at the C-terminus (Yang et al., 2005) (Figure 18-29). The inhibition can be annulled by Gα subunits, HSP90, or arachidonic acid which reorient the TPR domain and disrupt the contact with the catalytic domain.

The tetratricopeptide repeat (TPR) is a 34-amino acid-helical motif that has been identified in more than 300 proteins, whose functions range from cell-cycle control to transcriptional regulation, protein transport, protein folding, and neurogenesis. The repeats function as molecular scaffolds, frequently mediating protein–protein interactions. Individual TPR motif stack over each other, between 3 and 16, in a given protein domain. The regular, elongated structure that results has been likened to a spiral staircase in which the individual TPR motifs are the steps.

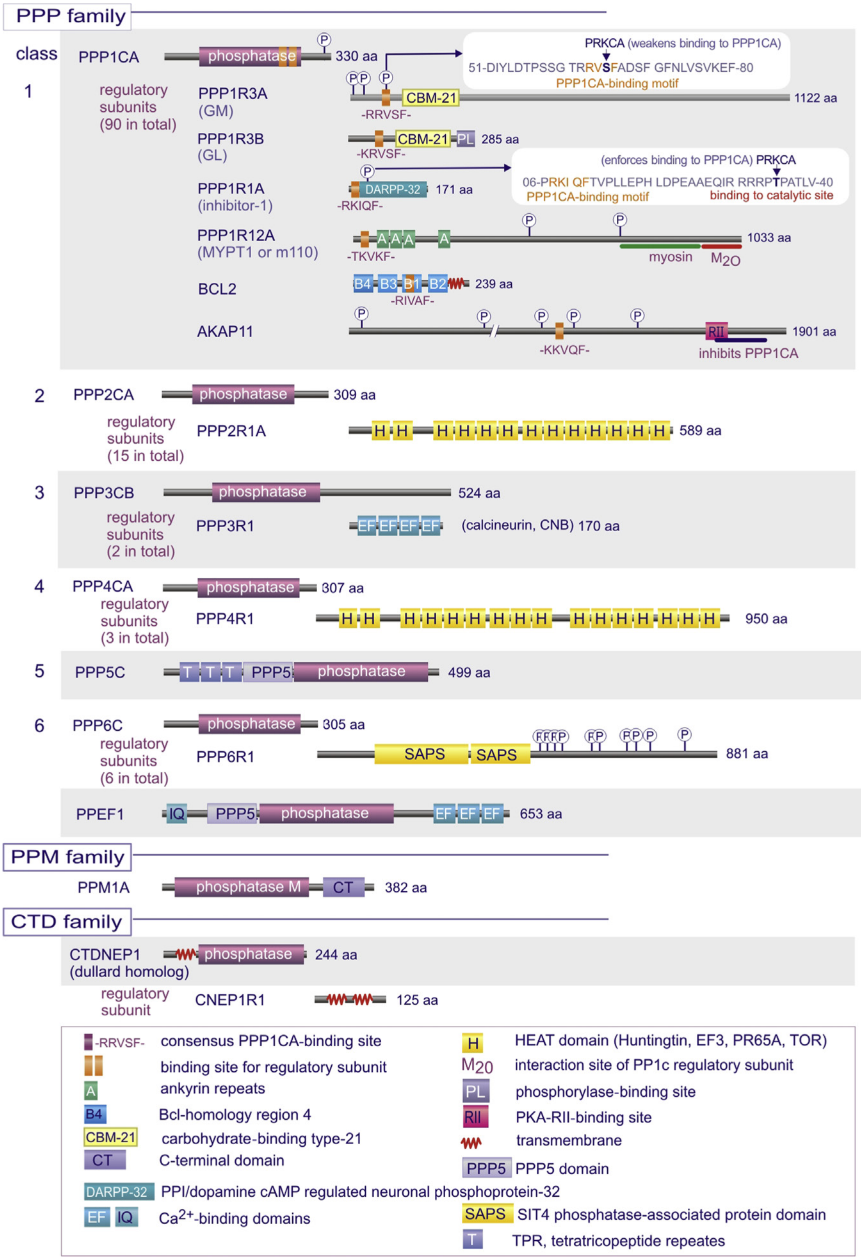

PPP family
class
1
PPP1CA
phosphatase
330 aa
PRKCA (weakens binding to PPP1CA)
51-DIYLDTPSSG TRRVSFADSF GFNLVSVKEF-80
PPP1CA-binding motif
regulatory subunits (90 in total)
PPP1R3A (GM)
CBM-21
-RRVSF-
1122 aa
PPP1R3B (GL)
CBM-21
PL
285 aa
-KRVSF-
(enforces binding to PPP1CA) PRKCA
06-PRKI QFTVPLLEPH LDPEAAEQIR RRRPTPATLV-40
PPP1CA-binding motif
binding to catalytic site
PPP1R1A (inhibitor-1)
DARPP-32
171 aa
-RKIQF-
PPP1R12A (MYPT1 or m110)
A A A A
1033 aa
-TKVKF-
myosin
M20
BCL2
B4 B3 B1 B2
239 aa
-RIVAF-
AKAP11
1901 aa
-KKVQF-
RII
inhibits PPP1CA
P
2
PPP2CA
phosphatase
309 aa
regulatory subunits (15 in total)
PPP2R1A
H H H H H H H H H H H H H H H
589 aa
3
PPP3CB
phosphatase
524 aa
regulatory subunits (2 in total)
PPP3R1
EF EF EF EF
(calcineurin, CNB) 170 aa
4
PPP4CA
phosphatase
307 aa
regulatory subunits (3 in total)
PPP4R1
H H H H H H H H H H H H H H H H H
950 aa
5
PPP5C
T T T PPP5 phosphatase
499 aa
6
PPP6C
phosphatase
305 aa
regulatory subunits (6 in total)
PPP6R1
SAPS SAPS
881 aa
PPEF1
IQ PPP5 phosphatase EF EF EF
653 aa
PPM family
PPM1A
phosphatase M
CT
382 aa
CTD family
CTDNEP1 (dullard homolog)
phosphatase
244 aa
regulatory subunit
CNEP1R1
125 aa
-RRVSF- consensus PPP1CA-binding site
binding site for regulatory subunit
A ankyrin repeats
B4 Bcl-homology region 4
CBM-21 carbohydrate-binding type-21
CT C-terminal domain
DARPP-32 PPI/dopamine cAMP regulated neuronal phosphoprotein-32
EF IQ Ca2+-binding domains
H HEAT domain (Huntingtin, EF3, PR65A, TOR)
M20 interaction site of PP1c regulatory subunit
PL phosphorylase-binding site
RII PKA-RII-binding site
transmembrane
PPP5 PPP5 domain
SAPS SIT4 phosphatase-associated protein domain
T TPR, tetratricopeptide repeates

FIGURE 18-27 Domain architecture of serine/threonine protein phosphatases (PPP and PPM families). The serine/threonine phosphatases are subdivided into three groups, the PPP phosphatases with Mn^{2+}/Fe^{2+} in the catalytic site, the PPM phosphatases with Mg^{2+}/Mn^{2+} (M domain), and the aspartate-based protein phosphatases (CTD). Most of these enzymes interact with regulatory or targeting subunits, some of which cannot be dissociated from the catalytic subunit. PPP5C is the exception for which no regulatory subunits have been identified and activity is regulated by three tetratricopeptide (TPR)-repeat motifs. The regulatory

and targeting subunits of PPP1CA illustrated all possess a consensus PPP1CA-binding motif (RRVSF, KRVSF, RKIQF, TKVKF, or RIVAF). This is a short stretch that interacts with the catalytic domain at a site adjacent to the catalytic pocket. The sequence around the consensus-binding motif for PPP1R3A (G_M) and PPP1R1A (I-1) provides sites for regulation by a number of protein kinases. Of particular importance is the regulation by PRKCA (PKA) which phosphorylates G_M and causes detachment of PPP1CA. In the case of RRR1R1A (inhibitor-1), PRKCA phosphorylation augments its interaction with PPP1CA by creating a pseudo-substrate site which binds the catalytic pocket of the phosphatase.

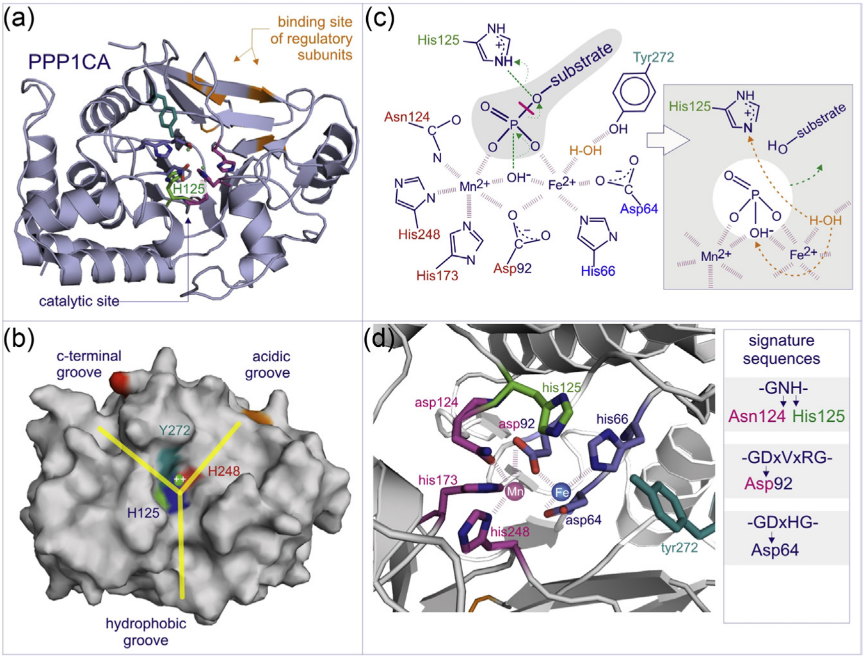


FIGURE 18-28 Members of the PPP family share a common catalytic domain structure. (a) The catalytic domain fold consists of a central β–sandwich surrounded on one side by seven α-helices and a three-stranded β-sheet. (b) The catalytic cleft has a Y-shape, with three branches commonly referred to as hydrophobic, acidic, and C-terminal grooves. (c) Crystallographic data provide compelling evidence for the role of two metal ions in the catalytic reaction. Most data are consistent with a single-step mechanism, employing a metal-activated nucleophilic water molecule or hydroxide ion. (d) The metal coordinating residues (asparagines, aspartates, and histidines) are invariant among the PPP subfamily members. The positions of some of the amino acids of the signature (conserved) sequences are shown in the right panel (Maynes et al., 2001).

Regulatory subunits of PPP1C

Members of PPP1C (A, B, or C) are widely distributed and regulates a broad range of cellular functions. These include glycogen metabolism, muscle relaxation (both smooth and skeletal muscle), and cell-cycle progression. The substrate specificities and the regulatory characteristics of

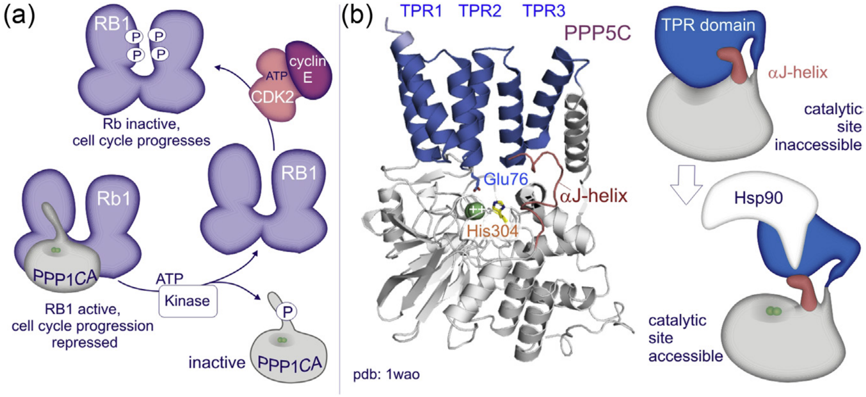


FIGURE 18-29 Regulation of PPP phosphatases through phosphorylation or intramolecular domain interaction. (a) Phosphorylation of the C-terminal region of PPP1CA hinders its catalytic activity toward the retinoblastoma protein (RB1). It is suggested that phosphorylation breaches the interaction between the two proteins, though other mechanisms could apply. Lack of phosphatase action leads to effective phosphorylation of RB1 by CDK2/CyclinE and allows the cell division cycle to proceed. (b) PPP5 is one of the PPP phosphatases that is not subject to regulation by subunits. Instead its inactive state is maintained by its TPR domain that wedges into the catalytic site. Glu76 and the hydrophobic αJ-helix are highlighted because they stabilize the autoinhibited state. The catalytic His304, adjacent to the Mn^{++}/Fe^{++}-binding site, is colored yellow. Proteins that interact with the TPR domain, such as HSP90, breach the interaction with the αJ-helix and expose the catalytic site.

muscle and liver type-1 phosphatases are quite distinct; yet the proteins are identical, their differences arising from association with different regulatory subunits (Cohen et al., 2006). About 90 PPP1C-binding proteins have been identified. They partake in different processes, and they localize the catalytic subunit to different subcellular compartments (Figure 18-30). Although the regulatory subunits are very dissimilar, most, but not all, do share a common PPP1C-binding sequence K/R-V/I-x-F (e.g., return to Figure 18-27).

Web resource

For a full view of all the regulatory subunits we refer to the HUGO gene-nomenclature database. Search for gene family, protein phosphatase, catalytic, and regulatory subunits. URL: www.genenames.org/genefamilies/PPP-PPM-CTD#PPP.

In smooth muscle, the regulatory subunit PPP1R12A (formerly MYPT1), targets PPP1CB to myosin and renders it more active against the myosin regulatory light chain. The main outcome of the interaction of PPP1R12A with PPP1CB is the formation of an extended acidic groove, well adapted

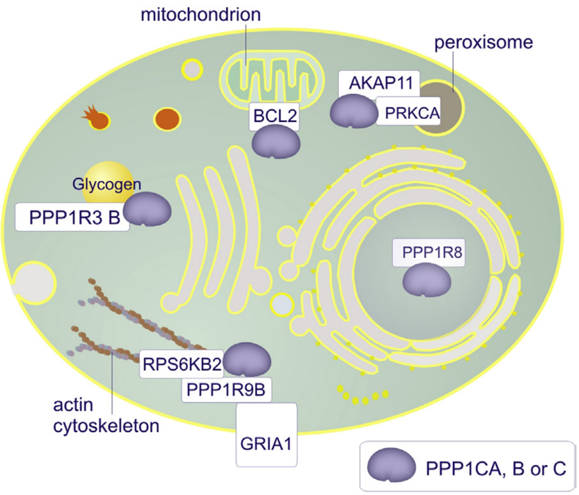


FIGURE 18-30 Regulatory subunits of members of the PPP1C subfamily. Regulatory subunits engage PPP1C (A, B, or C) in diverse metabolic events and localize the phosphatase to distinct subcellular compartments. A few examples of how regulatory subunits are involved in signaling, complex formation and the gathering of substrates, kinase and phosphatase, are illustrated.

to accommodate the basic N-terminal sequence of myosin regulatory light chain and making it less attractive for other substrates (Terrak et al., 2006) (Figure 18-31(a)).

Besides defining substrate specificity, regulatory subunits also allow the activity of PPP1C to be modulated by phosphorylation or by second messengers. For instance, RHOA-mediated regulation of smooth muscle contraction and the formation of focal adhesion contacts are exerted in part by RHOA-regulated kinase ROCK1. This phosphorylates the PPP1R12A-subunit, so suppressing PPP1CB activity toward the regulatory myosin light chain (RLC). Return to Chapter 3, “Regulation of Muscle Contraction by Adrenoceptors,” section “α1-Adreneceptors and visceral vasoconstriction.”

Small-molecule inhibitors of members of the PPP subfamily

PPP1C, PPP2C, PPP4C, and PPP5C are potently inhibited by the tumor promoters, okadaic acid, and microcystin LR (MacKintosh and MacKintosh, 1994). Figure 18-31, panel (b), shows how okadaic acid occupies a strategic position by hindering access of substrate through all three grooves. The complex PPP3CA/PPP3R1 (calcineurin) is inhibited by FK506 bound to FKBP1A (formerly FKBP12). Neither of these is capable of associating with the phosphatase alone, but together they form a stable system that inhibits its activity toward protein substrates while having no effect on the dephosphorylation of small model substrates (such as *p*-nitrophenol phosphate) (Griffith et al., 1995). In brain, PPP3CA forms a complex with

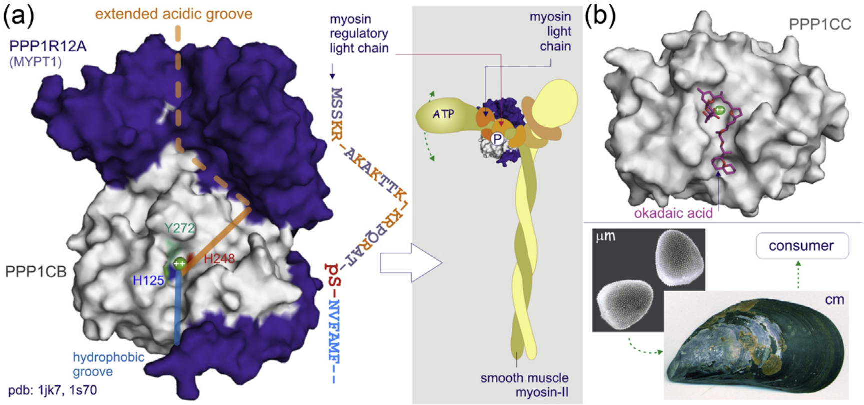


FIGURE 18-31 Regulation of PPP1CB by PPP1R12A. (a) Attachment of the regulatory subunit PPP1R12A (MYPT1) to PPP1CB (catalytic) creates an extended acidic groove which perfectly fits the N-terminal sequence of myosin regulatory light chain (myosin RLC) which holds many basic residues (K or R in orange). Moreover, a stretch of hydrophobic residues (in blue) preferably aligns the hydrophobic groove, positioning phosphoserine right on top of the catalytic pocket (green sphere). This explains how PPP1R12A (MYPT) increases the affinity (10×) of PPP1CB for this particular substrate. The phosphatase localizes near the myosin RLC on the heavy chain of smooth muscle myosin-II. Dephosphorylation of myosin RLC inactivates the ATPase activity of myosin-II and hence prevents movement of the head, resulting in muscle relaxation. (b) Okadaic acid, a causative toxin of diarrhetic shellfish poisoning in Europe, inhibits PPP1CC by occupying the catalytic cleft thereby preventing access of substrate. The toxin is produced by dinoflagellates and enters the food chain mainly through the consumption of bivalve mollusks.

AKAP5 (formerly AKAP79), an A-kinase anchoring protein that is a noncompetitive inhibitor of PPP3CA (Coghlan et al., 1995).

PPP1CA in the regulation of glycogen metabolism

Site-specific phosphorylation of the glycogen-targeting subunit PPP1R3A (G_M) of PPP1CA in muscle enables it to generate appropriate responses for adrenaline and Ca^{2+} on the one hand (glycogenolysis), and for insulin on the other (glycogen synthesis). In skeletal muscle, glycogenolysis (breakdown) is activated by adrenaline. It causes the production of cAMP leading to the activation of PRKCA (formerly PKA) (Figure 18-32(a)) and sets in train a number of phosphorylation reactions. First it phosphorylates G_M which destabilizes the complex by a factor of 10^4, allowing the dissociation of PPP1CA (Johnson et al., 1996). PRKCA also phosphorylates PPP1R1A (inhibitor-1) and through this it creates a pseudo-substrate-binding site (details in Figure 18-27). As a result, the liberated PPP1CA is hidden, unable to dephosphorylate proteins associated with glycogen. Finally, PRKCA phosphorylates

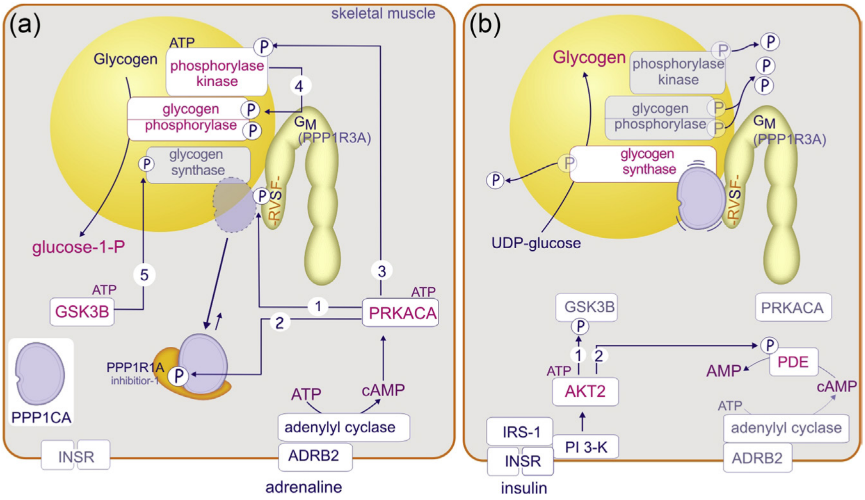


FIGURE 18-32 Decision making in glycogen synthesis and breakdown: opposing effects of adrenaline and insulin. (a) Adrenaline drives glycogenolysis through the activation of PRKACA. This leads to phosphorylation of PPP1R3A (G_M) in the PPP1CA-binding motif (-RVSF-, see Figure 18-27), causing them to dissociate (1). It also leads to phosphorylation of PPP1R1A (inhibitor-1) which, through the creation of a pseudo-substrate site, more effectively sequesters free PPP1CA (2). Finally PRKACA phosphorylates and activates glycogen phosphorylase kinase (3) which phosphorylates and activates glycogen phosphorylase (4). With the phosphorylation and inhibition of glycogen synthase by GSK3B (5), the balance shifts toward glycogenolysis with liberation of glucose-1-P. (b) Insulin stimulates glycogen synthesis. This is achieved by silencing the above protein kinases. AKT2 is activated and this phosphorylates and inactivates GSK3B (1). It also phosphorylates and activates phosphodiesterase (PDE) thereby depleting cAMP. With both GSK3B and PRKACA turned off, PPP1CA now dominates the scene. It inactivates both phosphorylase kinase and glycogen phosphorylase and it activates glycogen synthase. The balance shifts toward glycogen synthesis (incorporation of glucose).

and stimulates glycogen phosphorylase kinase. Because of the lack of PPP1CA activity, kinase activity now predominates. Phosphorylase kinase effectively phosphorylates and activates glycogen phosphorylase while simultaneously GSK3B phosphorylates and inactivates glycogen synthase. The consequence of all this is glycogenolysis, the net breakdown of glycogen into numerous units of glucose-1-phosphate.

Insulin brings about the reverse reaction (Figure 18-32(b)). It causes activation of PI 3-K which in turn leads to activation of AKT2. This phosphorylates and inactivates GSK3B (return to Chapter 16, “Signaling through the Insulin Receptor Phosphoinositide 3-Kinases and AKT,” Figure 16-11) and cAMP phosphodiesterase (PDE). As a result, the level of cAMP is suppressed and as a consequence PRKCA activity is suppressed. With the lack of both GSK3B and PRKCA activity, the phosphatase action of PPP1CA predominates, phosphorylase kinase and glycogen phosphorylase are inactivated, and glycogen synthase is activated. The balance shifts toward glycogen synthesis, with UDP-glucose units being added to the existing core of glycogen.

In order to switch from glycolysis to glycogen synthesis, PPP1CA is recruited to its regulatory G_M subunit. This requires the action of PPP2C or PPP3C to dephosphorylate both G_M and inhibitor-1 (Figure 18-33). This is another example of phosphatases acting as true reset buttons (Hubbard and Cohen, 1993).

The mechanisms that control phosphatase (PPP1CA) activity in liver and muscle are different. In liver, the activity of the G_L-subunit (PPP1R3B) is not controlled by phosphorylation. Instead, inhibition of phosphatase activity, necessary to suppress the action of glycogen synthase, is exerted by the phosphorylated (active) form of the target enzyme glycogen phosphorylase (Hubbard and Cohen, 1993) (Figure 18-34). This acts as an allosteric inhibitor of PPP1CA at extremely low concentrations. The interaction between the two is made possible because of the fact that G_L binds both the enzymes, PPP1CA and glycogen phosphorylase. This mechanism of gathering both enzymes together does not operate for the much larger G_M. Again, this provides an effective means for coupling the activation of glycogenolysis to the suppression of glycogen synthesis, and vice-versa.

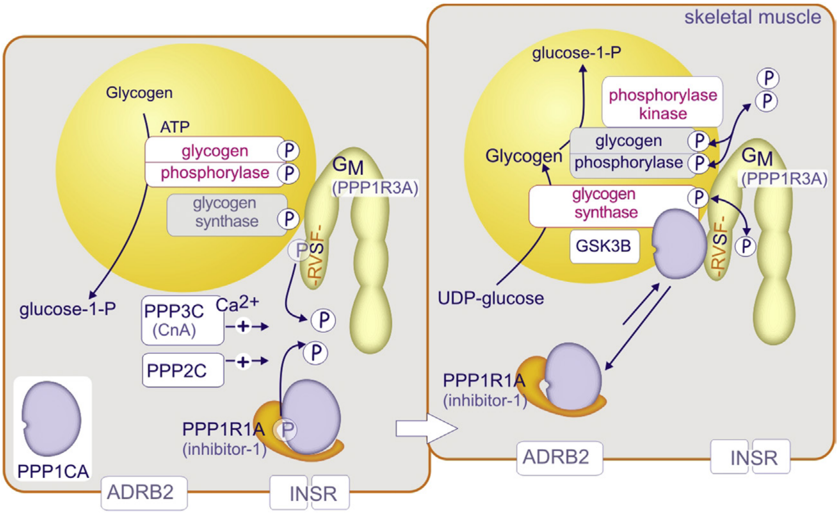


FIGURE 18-33 Rebalancing glycogen breakdown (PPP2C and PPP3C). When adrenaline is removed, glycogen metabolism is reset through dephosphorylation of the G_M-regulatory subunit. This is mediated by PPP2C and Ca^{2+}-dependent PPP3C (calcineurin). As a consequence PPP1CA rejoins G_M and glycogen metabolism again becomes sensitive to adrenaline and insulin. Under these "resting" conditions, it is likely that PPP1CA shuttles between G_M and inhibitor-1, allowing a low basal release of glucose-1-P.

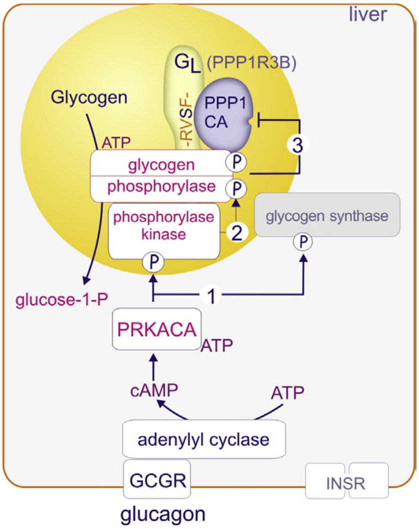


FIGURE 18-34 Regulation of glycogenolysis in liver: allosteric inhibition of PP1c by activated phosphorylase. In liver, both glycogen phosphorylase and PPP1CA bind the regulatory subunit G_L (distinct from G_M in muscle). When glycogen phosphorylase is activated, it inhibits the neighboring PPP1CA. Besides, PRKCA also phosphorylates and inactivates glycogen synthase which now remains phosphorylated due to the inhibition PPP1CA. Glycogen breakdown ensues.

PPP3C (calcineurin)

Although we now recognize that PPP3C has a wide tissue distribution (Chantler, 1985), it is identical to a Ca^{2+}-binding protein that was originally identified in neural tissue and named calcineurin (Klee et al., 1979). Sometime after its discovery, it was realized that calcineurin possesses phosphatase activity and that one of its regulatory subunits is the Ca^{2+}-binding protein calmodulin. The functional protein actually consists of three subunits: a catalytic subunit, calcineurin A (PPP3C), and two regulatory subunits calcineurin B (PPP3R1) and calmodulin. The principal role of PPP3R1 (CnB) appears to be structural. The phosphatase activity of PPP3C (CnA) is activated by Ca^{2+} as described in Chapter 15, "Activating the Adaptive Immune System: Role of Non-receptor Tyrosine Kinases."

Dephosphorylation of NFAT transcription factors: immunophilins show the way

Functional roles of calcineurin have come to light through the use of drugs that bind to cytosolic proteins called immunophilins. This name reflects the circumstances of their discovery. In 1972, ciclosporin A, a fungal product, was found to have remarkable immunosuppressive properties and few side effects (Borel et al., 1976). It is effective in preventing transplant rejection and graft versus host disease. Indeed, since its discovery, the use of ciclosporin A has enormously increased the life expectancy of patients receiving kidney, heart, and liver transplants. It also finds application in the treatment of autoimmune diseases and asthma (Sihra et al., 1997). It was nearly 10 years before the cellular target of ciclosporin A was identified as the cytosolic protein cyclophilin A (CypA). Later, another more potent immunosuppressive drug of fungal origin, FK506, was discovered. It targets the cytosolic-binding protein FKBP1A. CypA and FKBP1A are called immunophilins and are members of large protein families. The complexes of both FK506 and ciclosporin A with their respective target proteins inhibit calcineurin by hindering substrate access (McKeon, 1991) (Figure 18-35).

FK506, a macrolide derived from *Streptomyces* inhibits peptidyl–prolyl isomerase activity by binding to the immunophilin FKBP1A (FK506-binding protein). The FKBP1A–FK506 complex interacts with and inhibits calcineurin thus inhibiting both T-lymphocyte signal transduction and IL2 transcription. FK506 is widely used in the prevention of organ rejection after transplant surgery. The activity is similar to ciclosporin.

One important consequence of the inhibition of calcineurin is the failure of T-cell activation after engagement of the TCR with antigen, presented in

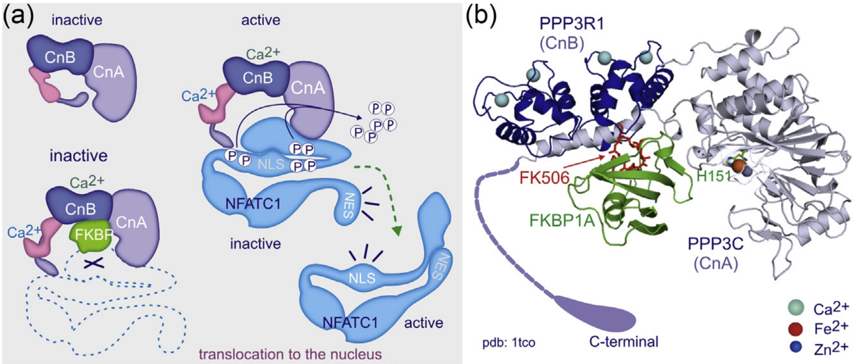


FIGURE 18-35 Immunophilins inhibit the phosphatase activity of calcineurin. (a) Under resting conditions calcineurin is in an auto-inhibited state in which the C-terminus interferes with the access of substrate. Antigen stimulation causes an increase in cytosolic free Ca^{2+} which binds to the regulatory domains PPP3R1 (CnB) and calmodulin (pink), thereby making the catalytic site of CnA available for substrate. In the context of T-cell activation, an important substrate is the transcription factor NFATC1, from which numerous phosphates are removed. This exposes the nuclear localization signal, leading to translocation into the nucleus. FK506 causes attachment of FKBP1A to the calcineurin complex and this blocks phosphatase activity. (b) The binding of FK506 to the calcineurin complex requires the simultaneous presence of PPP3C (CnA), PPP3R1 (CnB), and FKBP1A. Note that FKBP1A does not alter the catalytic site; it merely blocks access for large substrates. Small (designed) phosphopeptides still have access and are effectively dephosphorylated. Ca^{2+}, bound to CnB, and Fe^{2+} and Zn^{2+}, bound to CnA, are represented as colored spheres (Griffith et al., 1995).

MHC context (return to Chapter 15, Figure 15-2). This arises from the failure of calcineurin to dephosphorylate NFAT transcription factors in preparation for transfer into the nucleus. Inhibition by ciclosporin or FK506 causes an abrupt arrest of the TCR signal (Liu et al., 1991) and suppression of the immune response.

References

Abram, C.L., Roberge, G.L., Pao, L., Neel, B.G., Lowell, C.A., 2013. Distinct roles for neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice. Immunity 38, 489–501.

Ahmad, F., Azevedo, J.L., Cortright, R., Dohm, G.L., Goldstein, B.J., 1997. Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. J. Clin. Invest. 100, 449–458.

Alessi, D.R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S.M., Cohen, P., 1995. Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr. Biol. 55, 283–295.

Alessi, D.R., Kozlowski, M.T., Weng, Q.P., Morrice, N., Avruch, J., 1998. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Curr. Biol. 8, 69–81.

Alexander, D.R., 2000. The CD45 tyrosine phosphatase: a positive and negative regulator of immune cell function. Semin. Immunol. 12, 349–359.

Altan-Bonnet, G., Germain, R.N., 2005. Modeling T cell antigen discrimination based on feedback control of digital ERK responses. PLoS Biol. 3, e356.

Andersen, J.N., Mortensen, O.H., Peters, G.H., Drake, P.G., Iversen, L.F., Olsen, O.H., Jansen, P.G., Andersen, H.S., Tonks, N., 2001. Structural and evolutionary relationships among protein tyrosine phosphatase domains. Mol. Cell Biol. 21, 7117–7136.

Andersen, J.N., Jansen, P.G., Mortensen, O.H., Fukada, T., Del Vecchio, R., Tonks, N.K., Moller, N.P., 2004. A genomic perspective on protein tyrosine phosphatases: gene structure, pseudogenes, and genetic disease linkage. FASEB J. 18, 8–30.

Arkinstall, S., Nichols, A., 2000. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J. 14, 6–16.

Arnold, R.S., Shi, J., Murad, E., Whalen, A.M., Sun, C.Q., Polavarapu, R., Parthasarathy, S., Petros, J.A., Lambeth, J.D., 2001. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc. Natl. Acad. Sci. U.S.A. 98, 5550–5555.

Assoian, R.K., 1997. Control of the G1 phase cyclin-dependent kinases by mitogenic growth factors and the extracellular matrix. Cytokine Growth Factor Rev. 8, 165–170.

Baas, A.S., Berk, B.C., 1995. Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. Circ. Res. 77, 29–36.

Barr, A.J., Ugochukwu, E., Lee, W.H., King, O.N.F., Filippakopoulos, P., Alfano, I., Savitsky, P., Burgess-Brown, N.A., Muller, S., Knapp, S., 2009. Large-scale structural analysis of the classical human protein tyrosine posphatome. Cell 136, 352–363.

Barrett, W.C., DeGnore, J.P., Konig, S., Fales, H.M., Keng, Y.F., Yim, M.B., Chock, P.B., 1999. Regulation of PTP1B via glutathionylation of the active site cysteine 215. Biochemistry 38, 6699–6705.

Blanchetot, C., Tertoolen, L.G., den Hertog, J., 2002. Regulation of receptor protein-tyrosine phosphatase alpha by oxidative stress. EMBO J. 21, 493–503.

Borel, J.F., Feurer, C., Gubler, H.U., Stahelin, H., 1976. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions 6, 468–475.

Brady-Kalnay, S.M., Tonks, N.K., 1994. Identification of the homophilic binding site of the receptor protein tyrosine phosphatase PTPm. J. Biol. Chem. 269, 28472–28477.

Brondello, J.M., McKenzie, F.R., Sun, H., Tonks, N.K., Pouyssegur, J., 1995. Constitutive MAP kinase phosphatase (MKP-1) expression blocks G1 specific gene transcription and S-phase entry in fibroblasts. Oncogene 10, 1895–1904.

Brondello, J.M., Pouyssegur, J., McKenzie, F.R., 1999. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286, 2514–2517.

Bruskin, A.M., Johnson, K.A., Hill, D.E., 1992. Effect of protein tyrosine phosphatase 1B expression on transformation by the human neu oncogene. Cancer Res. 15, 478–482.

Byth, K.F., Conroy, L.A., Howlett, S., Smith, A.J., May, J., Alexander, D.R., Holmes, N., 1996. CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. J. Exp. Med. 183, 1707–1718.

Campbell, R.B., Liu, F., Ross, A.H., 2003. Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. J. Biol. Chem. 278, 33617–33620.

Campos-Ortega, J.A., Hartenstein, V., 1984. Early neurogenesis in wildtype *Drosophila melanogaster*. Roux's Arch. Dev. Biol. 193, 308–325.

Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., Arkinstall, S., 1998. Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. Science 280, 1212–1213.

Camps, M., Nichols, A., Arkinstall, S., 2000. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J. 4 (1), 6–16.

Cantley, L.C., Neel, B.G., 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 4240–4245.

Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., Clarkson, B., 1997. p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell 88, 197–204.

Chantler, P.D., 1985. Calcium-dependent association of a protein complex with the lymphocyte plasma membrane: probable identity with calmodulin-calcineurin. J. Cell Biol. 101, 207–216.

Charbonneau, H., Tonks, N.K., Kumar, S., Diltz, C.D., Harrylock, M., Cool, D.E., Krebs, E.G., Fischer, E.H., Walsh, K.A., 1989. Human placenta protein-tyrosine-phosphatase: amino acid sequence and relationship to a family of receptor-like proteins. Proc. Natl. Acad. Sci. U.S.A. 86, 5252–5256.

Charles, C.H., Abler, A.S., Lau, L.F., 1992. cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. Oncogene 7, 187–190.

Chen, H., Wertheimer, S., Lin, C.H., Katz, S.L., Amrein, K.E., Burn, P., Quon, M.J., 1997. Protein-tyrosine phosphatases PTP1B and syp are modulators of insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. J. Biol. Chem. 272, 8026–8031.

Cheng, A., Uetani, N., Simoncic, P.D., Chaubey, V.P., Lee-Loy, A., McGlade, C.J., Kennedy, B.P., Tremblay, M.L., 2002. Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B. Dev. Cell 2, 497–503.

Chiarugi, P., Cirri, P., 2003. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. Trends Biochem. Sci. 28, 509–514.

Cicirelli, M.F., Tonks, N.K., Diltz, C.D., Weiel, J.E., Fischer, E.H., Krebs, E.G., 1990. Microinjection of a protein-tyrosine-phosphatase inhibits insulin action in Xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A. 87, 5514–5518.

Coghlan, V.M., Perrino, B.A., Howard, M., Langeberg, L.K., Hicks, J.B., Gallatin, W.M., Scott, J.D., 1995. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science 267, 108–111.

Cohen, P.T., Schelling, D.L., da Cruz e Silva, O.B., Barker, H.M., Cohen, P., 2006. The major type-1 protein phosphatase catalytic subunits are the same gene products in rabbit skeletal muscle and rabbit liver. Biochim. Biophys. Acta 1008, 125–128.

Cohen, P., 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58, 453–508.

Cohen, P.T., 1994. Nomenclature and chromosomal localization of human protein serine/threonine phosphatase genes. Adv. Prot. Phosphat. 8, 371–376.

Cohen, P.T., 1997. Novel protein serine/threonine phosphatases: variety is the spice of life. Trends Biochem. Sci. 22, 245–251.

Coles, C.H., Mitakidis, N., Zhang, P., Elegheert, J., Lu, W., Stoker, A.W., Nakagawa, T., Craig, A.M., Jones, E.Y., Aricescu, A.R., 2014. Structural basis for extracellular cis and trans RPTP sigma signal competition in synaptogenesis. Nat. Commun. 5, 5209.

Cori, G.T., Cori, C.F., 1945. The enzymatic conversion of phosphorylase a to b. J. Biol. Chem. 158, 321–332.

Daigle, I., Yousefi, S., Colonna, M., Green, D.R., Simon, H.U., 2002. Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils. Nat. Med. 8, 62–67.

Denu, J.M., Tanner, K.G., 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry 37, 5633–5642.

Desai, D.M., Sap, J., Schlessinger, J., Weiss, A., 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. Cell 73, 541–554.

Digilio, M.C., Conti, E., Sarkozy, A., Mingarelli, R., Dottorini, T., Marino, B., Pizzuti, A., Dallapiccola, B., 2002. Grouping of multiple-lentigines/LEOPARD and Noonan syndromes on the PTPN11 gene. Am. J. Hum. Genet. 71, 389–394.

Dixon, J.E., Rhodes, J.D., 1992. Expression of a protein tyrosine phosphatase in normal and v-src-transformed mouse 3T3 fibroblasts. J. Cell Biol. 117, 401–414.

Dohadwala, M., da Cruz e Silva, E.F., Hall, F., Williams, R.T., Carbonaro-Hall, D.A., Nairn, A.C., Berndt, N., 1994. Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. Proc. Natl. Acad. Sci. U.S.A. 91, 6408–6412.

Dorfman, K., Carrasco, D., Gruda, M., Ryan, C., Lira, S.A., Bravo, R., 1996. Disruption of the erp/mkp-1 gene does not affect mouse development: normal MAP kinase activity in ERP/MKP-1-deficient fibroblasts. Oncogene 13, 925–931.

Dube, N., Cheng, A., Tremblay, M.L., 2004. The role of protein tyrosine phosphatase 1B in Ras signaling. Proc. Natl. Acad. Sci. U.S.A. 101, 1834–1839.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A.L., Normandin, D., Cheng, A., Himms-Hagen, J., Ramachandran, C., Gresser, M.J., Tremblay, M.L., Kennedy, B.P., 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283, 1544–1548.

Farooq, A., Zhou, M.M., 2004. Structure and regulation of MAPK phosphatases. Cell Signal. 16, 769–779.

Fischer, E.H., Charbonneau, H., Tonks, N.K., 1991. Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. Science 253, 401–406.

Flint, A.J., Tiganis, T., Barford, D., Tonks, N.K., 1997. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. Proc. Natl. Acad. Sci. U.S.A. 94, 1680–1685.

Goldstein, B.J., Wu, X., Zilbering, A., Zhu, L., Lawrence, J.T., 2001. Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. J. Biol. Chem. 28, 48662–48669.

Griffith, J.P., Kim, J.L., Kim, E.E., Sintchak, M.D., Thomson, J.A., Fitzgibbon, M.J., Fleming, M.A., Caron, P.R., Hsiao, K., Navia, M.A., 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell 82, 507–522.

Groom, L.A., Sneddon, A.A., Alessi, D.R., Dowd, S., Keyse, S.M., 2006. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J. 15, 3621–3632.

Guan, K.L., Broyles, S.S., Dixon, J.E., 1991. A Tyr/Ser protein phosphatase encoded by vaccinia virus. Nature 350, 359–362.

Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M.J., Shoelson, S.E., 1998. Crystal structure of the tyrosine phosphatase SHP-2. Cell 92, 441–450.

Hubbard, M.J., Cohen, P., 1993. On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem. Sci. 18, 172–177.

Irani, K., Xia, Y., Zweier, J.L., Sollott, S.J., Der, C.J., Fearon, E.R., Sundaresan, M., Finkel, T., Goldschmidt-Clermont, P.J., 1997. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 275, 1649–1652.

Irie-Sasaki, J., Sasaki, T., Matsumoto, W., Opavsky, A., Cheng, M., Welstead, G., Griffiths, E., Krawczyk, C., Richardson, C.D., Aitken, K., Iscove, N., Koretzky, G., Johnson, P., Liu, P., Rothstein, D.M., Penninger, J.M., 2001. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature 409, 349–354.

Ishii, K., Yamano, H., Yanagida, M., 1994. Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus and its potential role in cell cycle regulation. EMBO J. 13, 5310–5318.

Jiang, G., den Hertog, J., Su, J., Noel, J., Sap, J., Hunter, T., 1999. Dimerization inhibits the activity of receptor-like protein-tyrosine phosphatase-a. Nature 401, 606–610.

Johnson, D.F., Moorhead, G., Caudwell, F.B., Cohen, P., Chen, Y.H., Chen, M.X., Cohen, P.T., 1996. Identification of protein-phosphatase-1-binding domains on the glycogen and myofibrillar targetting subunits. Eur. J. Biochem. 239, 317–326.

Julien, S.G., Dube, N., Hardy, S., Tremblay, M.L., 2011. Inside the human cancer tyrosine phosphatome. Nat. Rev. Cancer 11, 35–49.

Kawakami, Y., Rodriguez-Leon, J., Koth, C.M., Buscher, D., Itoh, T., Raya, A., Ng, J.K., Esteban, C.R., Takahashi, S., Henrique, D., Schwarz, M.F., Asahara, H., Izpisua Belmonte, J.C., 2003. MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. Nat. Cell Biol. 5, 513–519.

Keilhack, H., David, F.S., McGregor, M., Cantley, L.C., Neel, B.G., 2005. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. J. Biol. Chem. 280, 30984–30993.

Kenner, K.A., Hill, D.E., Olefsky, J.M., Kusari, J., 1993. Regulation of protein tyrosine phosphatases by insulin and insulin-like growth factor I. J. Biol. Chem. 268, 25455–25462.

Keyse, S.M., 2000. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr. Opin. Cell Biol. 12, 186–192.

Kim, R.H., Peters, M., Jang, Y., Shi, W., Pintilie, M., Fletcher, G.C., DeLuca, C., Liepa, J., Zhou, L., Snow, B., Binari, R., Manoukian, A., Bray, M.R., Liu, F.F., Tsao, M.S., Mak, T.W., 2005. DJ-1, a novel regulator of the tumor suppressor PTEN. Cancer Cell 7, 263–273.

Kim, Y.C., Kitaura, H., Taira, T., Iguchi–Ariga, S.M., Ariga, H., 2009. Oxidation of DJ-1-dependent cell transformation through direct binding of DJ-1 to PTEN. Int. J. Oncol. 35, 1331–1341.

Kitamura, T., Feng, Y., Kitamura, Y.I., et al., 2006. Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nat. Med. 12, 534–540.

Klaman, L.D., Boss, O., Peroni, O.D., Kim, J.K., Martino, J.L., Zabolotny, J.M., Moghal, N.M., Lubkin, M., Kim, Y.B., Sharpe, A.H., Stricker-Krongrad, A., Shulman, G.I., Neel, B.G., Kahn, B.B., 2000. Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice. Mol. Cell Biol. 20, 5479–5489.

Klee, C.B., Crouch, T.H., Krinks, M.H., 1979. Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. U.S.A. 76, 6270–6273.

Knobbe, C.B., Lapin, V., Suzuki, A., Mak, T.W., 2008. The roles of PTEN in development, physiology and tumorigenesis in mouse models: a tissue-by-tissue survey. Oncogene 27, 5398–5415.

Kozomara, A., Griffiths-Jones, S., 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acid Res. 42, D68–D73.

Lassegue, B., Suh, Y.A., Arnold, R.S., Shi, J., Xu, X., Sorescu, D., Chung, A.B., Griendling, K.K., Lambeth, J.D., 1999. Cell transformation by the superoxide-generating oxidase Mox1. Nature 401, 79–82.

Lee, S.R., Kwon, K.S., Kim, S.R., Rhee, S.G., 1998. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J. Biol. Chem. 273, 15366–15372.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., Pavletich, N.P., 1999. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell 99, 323–334.

Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W., Rhee, S.G., 2002. Reversible inactivation of the tumor suppressor PTEN by H2O2. J. Biol. Chem. 277, 20336–20342.

Li, D.M., Sun, H., 1997. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res. 57, 2124–2129.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., Parsons, R., 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275, 1943–1947.

Li, S., Depetris, R.S., Barford, D., Chernoff, J., Hubbard, S.R., 2005. Crystal structure of a complex between protein tyrosine phosphatase 1B and the insulin receptor tyrosine kinase. Structure 13, 1643–1651.

Li, Q.-J., Chau, J., Ebert, P.J.R., Sylvester, G., Min, H., Liu, G., Braich, R., Manoharan, M., Soutschek, J., Skare, P., Klein, L.O., Davis, M.M., Chen, C.-Z., 2007. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell 129, 147–161.

Li, G., Yu, M., Lee, W.W., Tsang, M., Krishnan, E., Weyand, C.M., Goronzy, J.J., 2012. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nat. Med. 18, 1518–1524.

Li, Y.-F., Xu, S., Ou, X., Lam, K.-P., 2014. Shp1 signalling is required to establish the long-lived bone marrow plasma cell. Nat. Commun. 5, 4273.

Liu, J., Farmer Jr., J., Lane, W.S., Friedman, J., Weissman, I., Schreiber, S.L., 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66, 807–815.

Ma, W., Fuentes, G., Shi, X., Verma, C.S., Radda, G.K., Han, W., 2015. Foxo1 negatively regulates leptin-induced POMC transcription through its direct interaction with STAT3. Biochem. J. 466, 291–298.

MacKintosh, C., MacKintosh, R.W., 1994. Inhibitors of protein kinases and phosphatases. Trends Biochem. Sci. 19, 444–448.

Maehama, T., Dixon, J.E., 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. 273, 13375–13378.

Majeti, R., Xu, Z., Parslow, T.G., Olson, J.L., Daikh, D.I., Killeen, N., Weiss, A., 2000. An inactivating point mutation in the inhibitory wedge of CD45 causes lymphoproliferation and autoimmunity. Cell 103, 1059–1070.

Malaney, P., Pathak, Ramesh, Xu, B., Uversky, V.N., Dave, Vrushank, 2013. Intrinsic disorder in PTEN and its interactome confers structural plasticity and functional versatility. Nat. Sci. Rep. 3, 2035.

Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A.M., Martinez-Arias, A., 1998. Puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes Dev. 12, 557–570.

May, J.M., de Haen, C., 1979. The insulin-like effect of hydrogen peroxide on pathways of lipid synthesis in rat adipocytes. J. Biol. Chem. 254, 9017–9021.

Maynes, J.T., Bateman, K.S., Cherney, M.M., et al., 2001. Crystal structure of the tumor-promoter okadaic acid bound to protein phosphatase-1. J. Biol. Chem. 276, 44078–44082.

McGowan, C.H., Russell, P., 1993. Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. EMBO J. 12, 75–85.

McHeyzer-Williams, M., Okitsu, S., Wang, N., McHeyzer-Williams, L., 2012. Molecular programming of B cell memory. Nat. Rev. Immunol. 12, 24–34.

McKeon, F., 1991. When worlds collide: immunosuppressants meet protein phosphatases. Cell 66, 823–826.

Meng, T.C., Fukada, T., Tonks, N.K., 2002. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. Mol. Cell 9, 387–399.

Mukherjee, P., Lane, R.H., Lynn, W.S., 1978. Endogenous hydrogen peroxide and peroxidative metabolism in adipocytes in response to insulin and sulfhydryl reagents. Biochem. Pharmacol. 27, 2589–2594.

Myers, P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P., Tonks, N.K., 1998. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc. Natl. Acad. Sci. U.S.A. 95, 13513–13518.

Nagakubo, D., Taira, T., Kitaura, H., Ikeda, M., Tamai, K., Iguchi-Ariga, S.M., Ariga, H., 1997. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem. Biophys. Res. Commun. 231, 509–513.

Nagata, Y., Lan, K.H., Zhou, X., Tan, M., Esteva, F.J., Sahin, A.A., Klos, K.S., Li, P., Monia, B.P., Nguyen, N.T., Hortobagyi, G.N., Hung, M.C., Yu, D., Monia, B.P., Nguyen, N.T., Hortobagyi, G.N., Hung, M.C., Yu, D., 2004. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 6, 117–127.

Nam, H.J., Poy, F., Saito, H., Frederick, C.A., 2005. Structural basis for the function and regulation of the receptor protein tyrosine phosphatase CD45. J. Exp. Med. 201, 441–452.

Neel, B.G., Gu, H., Pao, L., 2003. The ‘Shp’ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem. Sci. 28, 284–293.

Noonan, J.A., 1968. Hypertelorism with Turner phenotype. A new syndrome with associated congenital heart disease. Am. J. Dis. Child. 116, 373–380.

Ohba, M., Shibanuma, M., Kuroki, T., Nose, K., 1994. Production of hydrogen peroxide by transforming growth factor-beta 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. J. Cell Biol. 126, 1079–1088.

Okumura, F., Matsuzaki, M., Nakatsukasa, K., Kamura, T., 2012. The role of elongin BC-containing ubiquitin ligases. Front. Oncol. 2, 10.

Ostman, A., Yang, Q., Tonks, N.K., 1994. Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density. Proc. Natl. Acad. Sci. U.S.A. 91, 9680–9684.

Pandit, B., Sarkozy, A., Pennacchio, L.A., Carta, C., Oishi, K., Martinelli, S., et al., 2007. Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat. Genet. 39, 1007–1012.

Patrick, A.N., Cabrera, J.H., Smith, A.L., Chen, X.S., Ford, H.L., Zhao, R., 2013. Structure-function analyses of the human SIX1-EYA2 complex reveal insights into metastasis and BOR syndrome. Nat. Struct. Mol. Biol. 20, 447–453.

Perkins, L.A., Larsen, I., Perrimon, N., 1992. Corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. Cell 70, 225–236.

Plum, L., Lin, H.V., Dutia, R., et al., 2009. The obesity susceptibility gene Cpe links FoxO1 signaling in hypothalamic pro-opiomelanocortin neurons with regulation of food intake. Nat. Med. 15, 1195–1201.

Pulido, R., Zuniga, A., Ullrich, A., 1998. PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. EMBO J. 17, 7337–7350.

Raabe, T., Riesgo-Escovar, J., Liu, X., Bausenwein, B.S., Maroy, P., Hafen, E., 1996. DOS, a novel pleckstrin homology domain-containing protein required for signal transduction between sevenless and Ras1 in Drosophila. Cell 85, 911–920.

Radhar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F., Devreotes, P.N., 2009. Phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. Proc. Natl. Acad. Sci. U.S.A. 106, 480–485.

Ravetch, J.V., Lanier, L.L., 2000. Immune inhibitory receptors. Science 290, 84–89.

Roach, T.I.A., Slater, S.E., White, L.S., Zhang, X., Majerus, P.W., Brown, E.J., Thomas, M.L., 1998. The protein tyrosine phosphatase SHP-1 regulates integrin-mediated adhesion of macrophages. Curr. Biol. 8, 1035–1039.

Ruivenkamp, C.A., van Weze, T., Zanon, C., Stassen, A., Vleck, C., Csikos, T., Klous, A.L., Tripdis, N., Perrak, S.A., Boerrighter, L., 2002. Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers. Nat. Genet. 31, 295–300.

Salmeen, A., Andersen, J.N., Myers, M.P., Tonks, N.K., Barford, D., 2000. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. Mol. Cell 6, 1401–1412.

Sap, J., Jiang, Y.P., Friedlander, D., Grumet, M., Schlessinger, J., 1994. Receptor tyrosine phosphatase R-PTP-k mediates homophilic binding. Mol. Cell Biol. 14, 1–9.

Sargin, B., Choudhary, C., Crosetto, N., Schmidt, M.H., Grundler, R., Rensinghoff, M., et al., 2007. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. Blood 110, 1004–1012.

Saxton, T.M., Ciruna, B.G., Holmyard, D., Kulkarni, S., Harpal, K., Rossant, J., Pawson, T., 2000. The SH2 tyrosine phosphatase shp2 is required for mammalian limb development. Nat. Genet. 24, 420–423.

Seo, M.S., Kang, S.W., Baines, I.C., Tekle, E., Chock, P.B., Rhee, S.G., 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. J. Biol. Chem. 272, 217–221.

Shannon, K., Bollag, G., 2007. Sending out an SOS. Nat. Genet. 39, 8–9.

Shen, W.H., Balajee, A.S., Wang, J., Wu, H., Eng, C., Pandolfi, P.P., Yin, Y., 2007. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell 128, 157–170.

Shi, Z.Q., Lu, W., Feng, G.S., 1998. The Shp-2 tyrosine phosphatase has opposite effects in mediating the activation of extracellular signal-regulated and c-Jun NH2-terminal mitogen-activated protein kinases. J. Biol. Chem. 273, 4904–4908.

Shi, Z.Q., Yu, D.H., Park, M., Marshall, M., Feng, G.S., 2006. Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity. Mol. Cell Biol. 20, 1526–1536.

Sihra, B.S., Kon, O.M., Durham, S.R., Walker, S., Barnes, N.C., Kay, A.B., 1997. Effect of cyclosporin A on the allergen-induced late asthmatic reaction. Thorax 52, 447–452.

Sluss, H.K., Han, Z., Barrett, T., Davis, R.J., Ip, Y.T., 1996. JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes Dev. 27, 45–58.

Song, M.S., Carracedo, A., Salmena, L., Song, S.J., Egia, A., Malumbres, M., Pandolfi, P.P., 2011. Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. Cell 144, 187–199.

Stralfors, P., Hiraga, A., Cohen, P., 1985. The protein phosphatases involved in cellular regulation. Purification and characterisation of the glycogen-bound form of protein phosphatase-1 from rabbit skeletal muscle. Eur. J. Biochem. 149, 295–303.

Sun, H., Tonks, N.K., 1994. The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. Trends Biochem. Sci. 19, 480–485.

Sun, H., Charles, C.H., Lau, L.F., Tonks, N.K., 1993. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 75, 487–493.

Sun, H., Tonks, N.K., Bar, S.D., 1994. Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. Science 266, 285–288.

Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., Finkel, T., 1995. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science 270, 296–299.

Takahashi, H., Graig, A.M., 2013. Protein tyrosine phosphatases PTPd, PTPs and LAR: presynaptic hubs for synapse organization. Trends Neurosci. 36, 522–534.

Tang, T.L., Freeman, R.M., O'Reilly, A.M., Neel, B.G., Sokol, S.Y., 1995. The SH2-containing protein-tyrosine phosphatase SH-PTP2 is required upstream of MAP kinase for early Xenopus development. Cell 80, 473–483.

Tanoue, T., Adachi, M., Moriguchi, T., 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat. Cell Biol. 2, 110–116.

Tarlinton, D., Radbruch, A., Hiepe, F., Dorner, T., 2008. Plasma cell differentiation and survival. Curr. Opin. Immunol. 20, 162–169.

Tartaglia, M., Mehler, E.L., Goldberg, R., Zampino, G., Brunner, H.G., Kremer, H., van der Burgt, I., Crosby, A.H., Ion, A., Jeffery, S., Patton, M.A., Kucherlapati, R.S., Gelb, B.D., 2001. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat. Genet. 29, 465–468.

Tartaglia, M., Kalidas, K., Shaw, A., Song, X., Musat, D.L., van der Burgt, I., Brunner, H.G., Bertola, D.R., Crosby, A., Ion, A., Kucherlapati, R.S., Jeffery, S., Patton, M.A., Gelb, B.D., 2002. PTPN11 mutations in Noonan syndrome: molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. Am. J. Hum. Genet. 70, 1555–1563.

Tartaglia, M., Niemeyer, C.M., Fragale, A., Song, X., Buechner, J., Jung, A., Hahlen, K., Hasle, H., Licht, J.D., Gelb, B.D., 2003. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat. Genet. 34, 146–150.

Tartaglia, T.A., 1997. The leptin receptor. J. Biol. Chem. 272, 6093–6096.

Terrak, M., Kerff, F., Langsetmo, K., Tao, T., Dominguez, R., 2006. Structural basis of protein phosphatase 1 regulation. Nature 429, 780–784.

Thareja, S., Aggarwal, S., Bhardwaj, T.R., Kumar, M., 2012. Protein tyrosine phosphatase 1B inhibitors: a molecular level legitimate approach for the management of diabetes mellitus. Med. Res. Rev. 32, 459–517.

Thomas, M.L., Barclay, A.N., Gagnon, J., Williams, A.F., 1985. Evidence from cDNA clones that the rat leukocyte-common antigen (T200) spans the lipid bilayer and contains a cytoplasmic domain of 80,000 Mr. Cell 41, 83–93.

Thomas, M.L., 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. Cell 58, 1055–1065.

Timofeev, O., Cizmecioglu, O., Settele, F., et al., 2010. Cdc25 phosphatases are required for timely assembly of CDK1-cyclin B at the G2/M transition. J. Biol. Chem. 285, 16978–16990.

Tonks, N.K., Charbonneau, H., Diltz, C.D., Fischer, E.H., Walsh, K.A., 1988a. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. Biochemistry 27, 8695–8701.

Tonks, N.K., Diltz, C.D., Fischer, E.H., 1988b. Purification of the major protein-tyrosine-phosphatases of human placenta. J. Biol. Chem. 263, 6722–6730.

Tonks, N.K., Cicirelli, M.F., Diltz, C.D., Krebs, E.G., Fischer, E.H., 1990. Effect of microinjection of a low-Mr human placenta protein tyrosine phosphatase on induction of meiotic cell division in Xenopus oocytes. Mol. Cell Biol. 10, 458–463.

Tonks, N.K., 2003. PTP1B: from the sidelines to the front lines!. FEBS Lett. 546, 140–148.

Tonks, N.K., 2006. Protein tyrosine phosphatases: from genes, to function, to disease. Nat. Rev. Mol. Cell Biol. 7, 833–846.

Touw, I.P., De Koning, J.P., Ward, A.C., Hermans, M.H., 2000. Signaling mechanisms of cytokine receptors and their perturbances in disease. Mol. Cell Endocrinol. 160, 1–9.

Trotman, L.C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N.P., Carver, B.S., Cordon-Cardo, C., Erdjument-Bromage, H., Tempst, P., Chi, S.G., Kim, H.J., Misteli, T., Jiang, X., Pandolfi, P.P., 2007. Ubiquitinylation regulates PTEN nuclear import and tumor suppression. Cell 128, 141–156.

Tsui, H.W., Siminovitch, K.A., de Souza, L., Tsui, F.W., 1993. Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. Nat. Genet. 4, 124–129.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., Wahl, G.M., 2002. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol. Cell 9, 1031–1044.

Varma, R., Campi, G., Yokosuka, T., et al., 2006. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. Immunity 25, 117–127.

Vazquez, F., Ramaswamy, S., Nakamura, N., Sellers, W.R., 2000. Phosphorylation of the PTEN tail regulates protein stability and function. Mol. Cell Biol. 20, 5010–5018.

Vazquez, F., Matsuoka, S., Sellers, W.R., Yanagida, T., 2006. Tumor suppressor PTEN acts through dynamic interaction with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 103, 3633–3638.

Wang, W., Liu, L., Song, X., Mo, Y., Komma, C., Bellamy, H.D., Zhao, Z.J., Zhou, G.W., 2011. Crystal structure of human protein tyrosine phosphatase SHP-1 in the open conformation. J. Cell Biochem. 112, 2062–2071.

Werner, E., Werb, Z., 2002. Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. J. Cell Biol. 158, 357–368.

Yamaguchi, H., Hendrickson, W.A., 1996. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature 384, 484–489.

Yamaguchi, Y., Katoh, H., Mori, K., Negishi, M., 2002. Ga12 and Ga13 interact with ser/thr protein phosphatase type 5 and stimulate its phosphatase activity. Curr. Biol. 12, 1353–1358.

Yang, J., Liang, X., Niu, T., Meng, W., Zhao, Z., Zhou, G.W., 1998. Crystal structure of the catalytic domain of protein-tyrosine phosphatase SHP-1. J. Biol. Chem. 273, 28199–28207.

Yang, J., Roe, S.M., Cliff, M.J., Williams, M.A., Ladbury, J.E., Cohen, P.T., Barford, D., 2005. Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. EMBO J. 24, 1–10.

Yoo, L.I., Pingel, J.T., Heikinheimo, M., Klemola, T., Varkila, K., Vuopala, K., Poyhonen, M., Rogers, M., Chatila, T., Thomas, M.L., 2000. Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. Nat. Med. 6, 343–345.

Yu, Z.H., Xu, J., Walls, C.D., Chen, L., Zhang, S., Zhang, R., Wu, L., Wang, L., Liu, S., Zhang, Z.Y., 2013. Structural and mechanistic insights into LEOPARD syndrome-associated SHP2 mutations. J. Biol. Chem. 288, 10472–10482.

Zhang, J., Somani, A.K., Siminovitch, K.A., 2000. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. Semin. Immunol. 12, 361–378.

Zhang, X.C., Piccini, A., Myers, M.P., Van Aelst, L., Tonks, N.K., 2012. Functional analysis of the protein phosphatase activity of PTEN. Biochem. J. 444, 457–464.

Zheng, X.M., Wang, Y., Pallen, C.J., 1992. Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. Nature 359, 336–339.

Zikherman, J., Doan, K., Parameswaran, R., Raschke, W., Weiss, A., 2012. Quantitative differences in CD45 expression unmask functions for CD45 in B-cell development, tolerance, and survival. Proc. Natl. Acad. Sci. U.S.A. 109, E3–E12.

Zimmerman, J.E., Bui, Q.T., Steingrimsson, E., Nagle, D.L., Fu, W., Genin, A., Spinner, N.B., Copeland, N.G., Jenkins, N.A., Bucan, M., Bonini, N.M., 1997. Cloning and characterization of two vertebrate homologs of the Drosophila eyes absent gene. Genome Res. 7, 128–141.

Zinker, B.A., Rondinone, C.M., Trevillyan, J.M., Gum, R.J., Clampit, J.E., Waring, J.F., Xie, N., Wilcox, D., Jacobson, P., Frost, L., Kroeger, P.E., Reilly, R.M., Koterski, S., Opgenorth, T.J., Ulrich, R.G., Crosby, S., Butler, M., Murray, S.F., McKay, R.A., Monia, B.P., Jirousek, M.R., 2002. PTP1B antisense oligonucleotide lowers PTP1B protein, normalizes blood glucose, and improves insulin sensitivity in diabetic mice. Proc. Natl. Acad. Sci. U.S.A. 99, 11357–11362.

CHAPTER

19

Cell Fate Determination by Notch

NOTCHED WINGS, MORGAN, AND THE GENE THEORY

The appearance of notches present in the wings of *Drosophila* was first described by Thomas Hunt Morgan in 1916, who ascribed them to a spontaneous genetic mutation (Figure 19-1). At that time, genes, the elements of heredity postulated by Gregor Mendel some 50 years previously, remained just that, postulates. Where they resided and what they were made of were entirely obscure. Morgan, an embryologist, not a geneticist by training, wanted to know how eggs of different species, that appear almost identical under the microscope, mature into organisms of almost unlimited variety. He appreciated that development and heredity are inextricably linked and that knowledge in this area could not be advanced without an understanding of the nature of the gene. As he soon found out, his genetically minded colleagues were more interested in cataloging phenotypic oddities, showing little interest in the nature of the gene. Thus it fell to him to tackle the central question and introduce the science of genetics into embryology, or, as he phrased it, "*approaching a physiological problem with a new and strange discipline to the classical physiology of the schools*." This, and perhaps the fact that they were cheap to work with, explains his affair with flies.

Web resource

In writing these paragraphs, we have relied heavily on the work of Scott Gilbert. see http://10e.devbio.com/. Further details can be found in Gilbert (1978, 1987).

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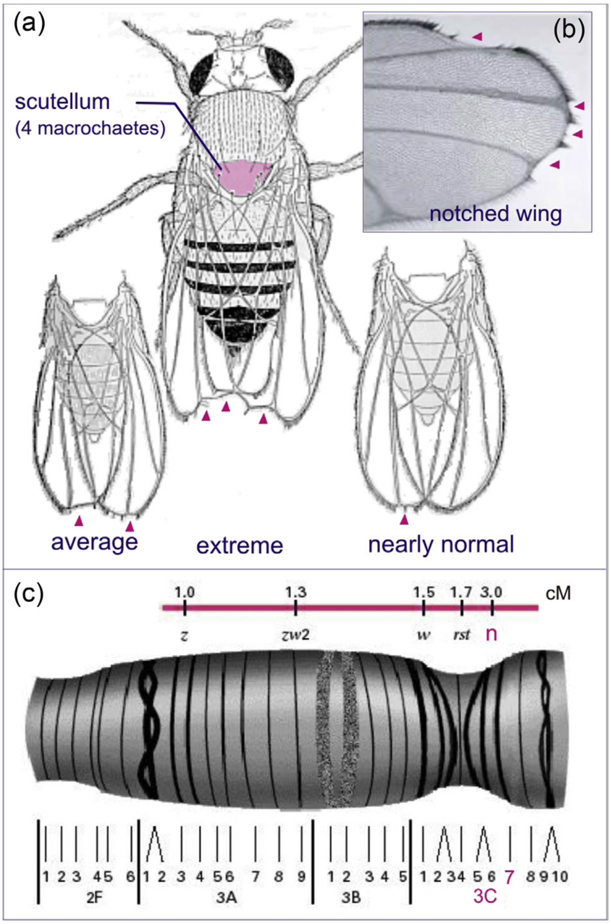

FIGURE 19-1 Appearance of the Notch phenotype. (a) Notched wings, first described by Morgan and drawn by Edith Wallace, artist and curator of the *Drosophila* stocks at Columbia University. *(From Morgan (1919))*. (b) A photograph of a notched wing. Note the intermittent absence of small sensory bristles (microchaete) along the edge. From "The interactive fly", Thomas B Brody, section "Genes involved in tissue and organ development." (c) Localization of the Notch locus on the polytene salivary X-chromosome of *Drosophila melanogaster*. z, zeste; zw2, zeste-white2; w, white; N, notch; *rst*, roughest. Locus distance in centimorgan (cM). 1cM (centimorgan) represents ~300 kB.

At that time, there were two dominant ideas concerning the way that single egg cells develop into organisms having a multitude of different organs. Some embryologists, perhaps those who had carefully observed the initial cleavage stages under the microscope, put it about that the answer must lie in the distribution of the protoplasm. Through a series of asymmetric divisions, certain cells contained, whereas others lacked the material required to constitute individual organs. They failed to consider how the information might be conserved and then transmitted through subsequent generations. Another school of thought had it that all the information resides in the nucleus. With time, through progressive reorganization of the nuclear material (chromatin), different sets of information would be retained in some cells, not in others, in this way guiding the different stages of embryonic development. This proposal was influenced by the ideas of Claude Bernard who had enunciated the principle that cells show metabolic specialization, the nucleus being the site of synthetic metabolism, and degradative processes confined to the cytoplasm. These "pronuclear" embryologists took this to mean that the nucleus operates in development by setting up a continuous series of specific metabolic changes in the cytoplasm leading to different cells that eventually constitute the various organs.

Initially Morgan sided with the cytosolists, denying the evidence for the "nuclear preformation" approach to development. The belief was founded, in part, on his own experiments which revealed the impotence of the nucleus when a segment of cytoplasm was removed: "*A defect in the protoplasm often brings about a modified cleavage and also a defective embryo, and this takes place even though the whole of the nuclear material of the unsegmented egg remains present. There seems, therefore no escape from the conclusion that in the protoplasm, and not in the nucleus, lies the differentiating power of the early stages of development.*"

Hans Driesch eventually revealed that the two opposing theories, progressive determination of the cytoplasm by the nucleus versus cytoplasmic control of nuclear function, are as the two sides of a coin. He showed, first by severing the blastomeres of sea urchin eggs and then later by altering their cleavage orientations, that the nucleus of a cell destined originally to become ectoderm can retain the ability to become endoderm or even to generate the entire embryo itself. Driesch correctly concluded that the nucleus and the cytoplasm interact to guide all the succeeding stages of development: "*Insofar as it carries a nucleus, every cell, during ontogenesis, carries the totality of all primordia; insofar as it contains a specific cytoplasmic cell body, it is specifically enabled by this to respond to specific effects only... When nuclear material is activated, then, under its guidance, the cytoplasm of the cell that had first influenced the nucleus is in turn itself changed, and thus the basis is established for a new elementary process, which itself is not only a result but also a cause.*"

Compelling evidence for nuclear control of the phenotype came from experiments designed to resolve the mechanism of sex determination. Studies of male gametogenesis in the lubber grasshopper, by Sutton and McClung, demonstrated that half of the spermatocytes carry an "accessory chromosome" that has an effect on sex determination following fertilization (although they wrongly linked this chromosome with development of the male sex). Henking had already noted this accessory chromosome in plants, and not knowing its function, labeled it "X," destined to become the identifier of the larger of the two universal sex chromosomes. Further evidence for a role of chromosomes in sex determination came from the work of Nettie Stevens who showed that all the somatic cells in female mealworm beetles possess 20 large chromosomes (18 + 2X) while those of the male have 19 large, plus 1 small one (18 + X + Y). The unfertilized eggs have 10 large (9 + X), and the sperm have either 10 large (9 + X) or nine large and one small chromosome (9 + Y). She called the process she observed a "*clear case of sex determination, not by an accessory chromosome but by a definite difference in the character of the elements of one pair of chromosomes of the spermatocyte of the first order, the spermatozoon which contains the small chromosome determining the male sex*."

All this conflicted with Morgan's views and in order to reach a resolution he commenced an investigation of sex-limited inheritance in *Drosophila*. In 1910, assisted by some white-eyed mutant flies that had entered his stocks by chance, he found that the inheritance of red eyes is inseparable from the X-chromosome. By the following summer, he had shown that the factors that determine eye color, body color, wing shape, and sex all segregate together with the X-chromosome.

To explain these drastic deviations from random assortment, he formulated a principle of association: "*By this I mean that during segregation, certain factors are more likely to remain together than to separate, not because of any attraction between them, but because they lie near together on the same chromosome*." This constituted direct evidence for the determination of sex by the chromosomes: he had provided evidence that genes are heritable elements localized on chromosomes in the nucleus and that their absence or presence affects the phenotype. This finding is known as the "gene theory."

Of course, the discovery of the nature of the genes was still 40 years off. Only in 1953 were Watson and Crick able to put forward the now generally accepted structure of DNA. In their paper, they also mentioned that "*it had not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material*," thereby, also solving the long-standing problem of how genetic information is stored and transmitted from generation to generation.

In 1913, Morgan and A.H. Sturtevant presented the first physical genetic map of the X-chromosome of *Drosophila*. With a continuous search of mutants and crossing experiments, genetic maps of other chromosomes came to light. The notched wing *Drosophila* played a part in all this.

By 1933, when Morgan received the Nobel Prize in Physiology or Medicine "for his discoveries concerning the role played by the chromosome in heredity," developmental biology had advanced, especially with the findings of Spemann and others. It was understood that environmental factors, operating by cell–cell contact or diffusible factors, influence cell fate (the discovery of the "organizer," return to Chapter 17, "TGFβ and Signaling through Receptor Serine/Threonine Protein Kinases," section “Sequestration of ligand and opposition by FGF prevent BMP signaling and causes dorsalization of Xenopus Laevis embryos”). But again the problem of how these interactions change the phenotype of cells remained unsolved. In his Nobel speech Morgan commented: *“From the point of view under consideration, results of this kind are of interest because they bring up once more, in a slightly different form, the problem as to whether the organizer acts first on the protoplasm of the neighboring region with which it comes in contact, and through the protoplasm of the cells on the genes; or whether the influence is more directly on the genes. In either case the problem under discussion remains exactly where it was before. The evidence from the organizer has not as yet helped to solve the more fundamental relation between genes and differentiation, although it certainly marks an important step forward in our understanding of embryonic development.”*

We now know that cell environmental factors, acting through receptors, operate signal transduction pathways that directly or indirectly (via a change in protein translation) affect the activity of transcription factors, leading to a change of gene transcription. The Notch signal transduction pathway is no exception to the general rule. It modifies cell fate by silencing some genes and inducing the expression of others, mediating an essential and perhaps universal function in the assignation of cell fates during development (Arias, 2002). Notch is just one example of how environment, in this case the neighboring cell, influences cell fate, but its mode of action provides a perfect and even simple answer to the question that Morgan had been asking throughout his career. Unlike any other system that we have discussed, it acts through ligand-induced cleavage of its own intracellular segment that moves straight to the nucleus there to act as a regulator of transcription.

ONE GENE, MANY ALLELES

The notched-wing *Drosophila* is the consequence of null alleles, abnormal genes that result in loss-of-function of the gene product. They are the consequence of nonsense mutations, a translocation or an inversion of the chromosome. Null alleles for Notch are lethal in hemizygous males (XY) and only viable in heterozygous females in which they are partially compensated by a functional gene. There are numerous lethal Notch alleles and they are indicated with an N plus extra superscript indicators N^{40}, N^{NIC}, etc. Females carrying one mutated N allele display the characteristic notched wings, thickened wing veins, and some bristle abnormalities. Many other Notch alleles have been detected which are not lethal, being the consequence of a partial loss of function due to missense mutations, partial deletions, or very low levels of expression. One group of nonlethal alleles, the “recessive visibles,” that fall in three complementation groups, facet (fa), split (spl), and notchoid (Nd), affect either wing or eye morphology. Yet another class comprises the dominant abruptex (Ax) mutations, which cause missense mutations in the extracellular (ligand binding) domain of Notch and, surprisingly, enhance the Notch signal.

When Poulson commenced his analysis of flies that had lost the whole or certain bands of the X-chromosome, among which the band that contains the Notch locus, he discovered a bewildering array of morphological abnormalities affecting nearly all parts of the body. An excess of neural tissue was particularly apparent. This provided first evidence that Notch has an important role in the early stages of development (Poulson, 1937, 1950). These were confirmed and extended by the work of Campos-Ortega (Hartenstein, 2006) who went on to demonstrate that the hypertrophy of the neural system is due to a defect that arises very early in development, roughly 4 h after fertilization. At this stage, neural precursor cells (neuroblasts) segregate from others that constitute the neurogenic ectoderm. Normally, only the neuroblasts give rise to neural tissue and the remainder of the neurogenic ectoderm turns into epidermis (Campus Ortega and Hartenstein, 1984). However, when Notch is missing, nearly all cells proceed toward a neural fate and very few are left to form the hypodermis. This was later explained by a lack of lateral inhibition (Doe and Goodman, 1985). We return to this phenomenon below. Here too Notch plays the role of suppressing the default neural fate thus ensuring that a sufficient number of proneural cells become epidermal to provide a strong cuticle. Other defects were found in the formation of somites (muscle), the Malpighian tubules (renal function), and the eyes. Using temperature-sensitive mutants (in which the loss of function can be induced by a change of temperature), anomalies were observed in the process of oogenesis, in which lack of Notch activity alters the composition of the egg chamber (excess of polar cells) (Simpson, 1994). Lastly, similar phenotypes occur with mutations at loci, Delta and Serrato, that harbor genes coding for the ligands of Notch. Below we will describe how Notch is activated and how the signal is transmitted to the nucleus, there to change gene expression and thus causing a switch in cell fate.

MEMBRANE COMPONENTS OF THE NOTCH PATHWAY

Notch ligands (DSL proteins)

The Notch ligands are single-pass transmembrane proteins that are characterized by an N-terminal DSL motif, named after representatives of the family of ligands present in three different organisms; Delta (Human), Serrate (*Drosophila*), and LAG-2 (*Xenopus*) (Figure 19-2).

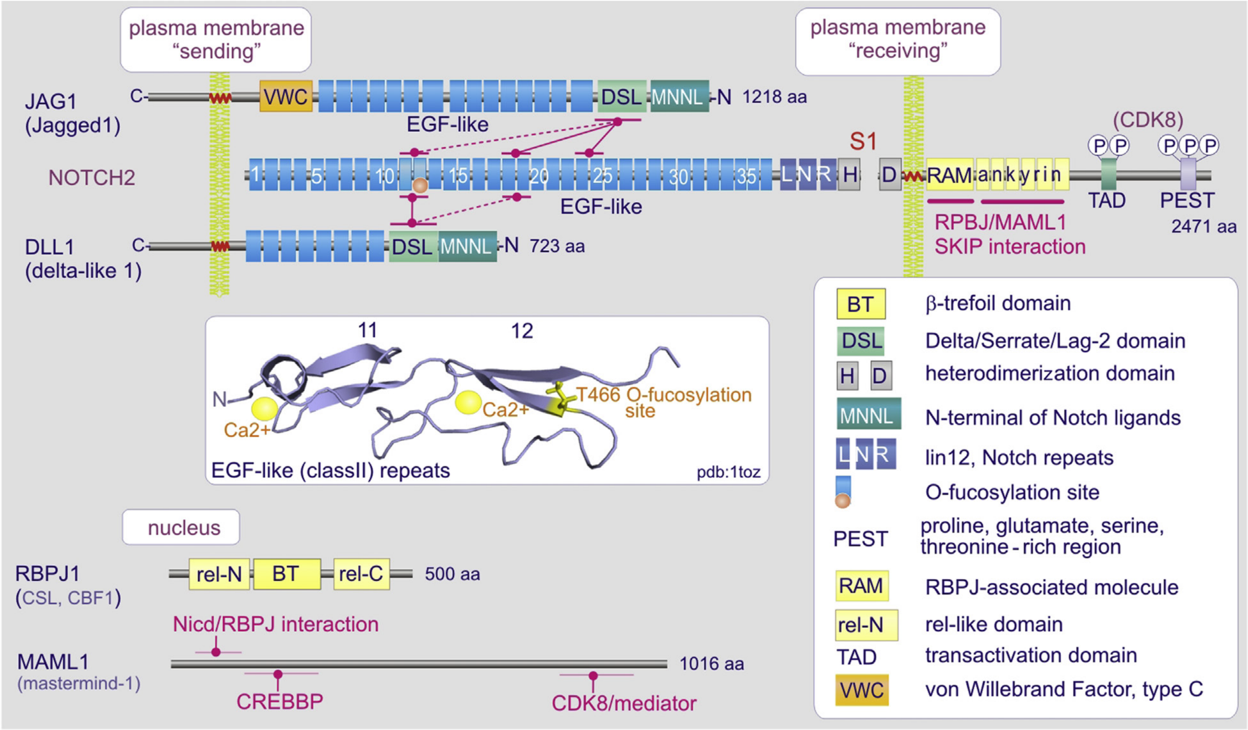

FIGURE 19-2 Domain architecture of Notch, its ligands, and nuclear proteins associated with its intracellular segment. Both Notch and its ligands contain numerous EGF-like domains. This renders them long and flexible but the flexibility is restrained at domains that bind Ca^{2+}. The DSL domains interact with Notch at different sites. The mature Notch protein is cleaved in the heterodimerization domain (S1). The intracellular segment contains a RAM domain, approximately 100 residues and loosely defined as a region that commences at the γ-secretase cleavage site (see Figure 19-3) and ends at the first ankyrin repeat. The C-terminal region contains TAD- and PEST motifs, both subject to phosphorylation by the protein kinase CDK8 (associated with the mediator complex). The nuclear protein RBPJ binds the intracellular fragment of Notch, in which both the BT and C-terminal Rel-like domains take part. Mastermind-1, which binds RBPJ and the intracellular Notch segment (Nicd) with its N-terminal regions, has no recognizable domains. PEST constitutes a proline-glutamate-serine-threonine-rich sequence.

Although this domain is essential for interactions with the receptor, molecular understanding of how ligand and receptor interact is still lacking. The ligands are separated into two classes; Delta (or Delta-like) and Serrate (or Jagged in mammals). The main structural difference between the two is an additional membrane proximal cysteine-rich region, resembling the von Willebrand factor type C (VWC) domain, in the Jagged members (Lissemore and Starmer, 1999). Mammals have five genes encoding their ligands, three Delta-like-1,3,4 and two Jagged-1,2 (DLL1, -3, -4 and JAG1, -2), *Drosophila* carries only one Delta and one Serrate, whereas *Caenorhabditis elegans* has several Delta homologs (lag-2, apx-1, Arg-1, DSL-1) (see Table 19-1).

Notch receptors

The Notch receptors are also single-pass transmembrane proteins occurring in the plasma membrane as heterodimeric molecules, comprising one extracellular and one transmembrane segment, linked noncovalently (although a disulfide bond has been suggested) (Figure 19-2). The fucosylated Notch precursor is further processed in the *trans*-Golgi by cleavage of the S1-site by a furin-like convertase (Logeat et al., 1998). This is essential for Notch activity in mammals, although *Drosophila* appears to get away with uncut receptors (Kidd and Lieber, 2002). The extracellular segment contains numerous (26–36) EGF-like domains and three cysteine-rich repeats (Lin-repeats) that mask an essential second extracellular cleavage site (S2) (Figure 19-3). As an indication of its importance, the region covering the Lin-repeats is altered in 26% of activating mutations that are associated with T-cell acute lymphoblastic leukemia (Weng et al., 2004) (see also Figure 19-18). The DSL domain of the ligand interacts with EGF-like repeats number 11 and 12 but other regions are not excluded (Figure 19-2). Some of the EGF-like repeats contain Ca^{2+}-binding sites, which stiffen the molecule (as in the cadherin adhesion molecules), whereas others are modified by fucosylation on serine or threonine (for instance, T466 on EGF-like repeat 12) (Hambleton et al., 2004). Mammals have four genes, Notch1, -2, -3, -4, *Drosophila* carries only copy, *C. elegans* has two (Table 19-1).

Functional Notch requires plasma membrane expression of the tetraspan membrane protein Sanpodo in *Drosophila* (gene name Spdo) (O'Connor-Giles and Skeath, 2003). Little is known about this protein, which was originally discovered as the homolog of the actin-associated tropomodulin. It acts downstream of Delta and upstream of Notch to which it binds. Importantly, loss of its expression at the plasma membrane with subsequent accumulation in endocytic vesicles, renders cells unresponsive to Delta. The functional human homolog of Sanpodo is NUMB-like (NUMBL) (only 13% sequence identity). We return to

TABLE 19-1 Components of the Notch signaling pathway

Mammals	*Drosophila*	*Caenorhabditis elegans*	Function
Transmembrane ligands and receptor			
NOTCH 1–4	Notch	Lin-12, Glp-1	Transmembrane receptor and transcription factor
DELTA1,3,4 JAG1,2 (jagged)	Delta, Serrate	APX-1, Lag-2, Arg-1, DSL-1	Transmembrane ligand of Notch receptor
Transcription (co)-factors			
RBPJ (CBF1/CSL)	Su(H)	Lag-1	DNA-binding transcription factor
MAML1–3 (mastermind-like)	Mastermind	Lag-3	Transcriptional coactivator
Receptor-associated proteins (involved in activity and endocytosis)			
NUMB	Numb	Num-1	Links Notch to α-adaptin (AP-2 component)
NUMBL	Sanpodo		Membrane localization strictly required for Notch activity (positive), links Notch with numb (negative)
Glycosylation enzymes of ligands and receptor			
POFUT1	Ofut1	Ofut1	GDP-fucose protein O-fucosyltransferase (modifies receptor and ligand, activates signaling)
LFNG (fringe)	Fringe	No homolog identified	O-fucosylpeptide β-1,3-N-acetylglucosaminyltransferase (modifies receptor and ligand, activates signaling)
Regulation of endocytosis			
DNM1, 2 (dynamin)	Shibire	Dyn-1	Required for pinching-off vesicle
DNAJC6 (auxilin)	Auxilin	Dnj-25	Role in clathrin uncoating (for further processing) clathrin-associated sorting protein, required for Delta signaling
EPN1–3 (epsin)	lqf (liquid facets)	Epn1	

Continued

TABLE 19-1 Components of the Notch signaling pathway —cont'd

Mammals	***Drosophila***	***Caenorhabditis elegans***	**Function**
Transmembrane proteases			
ADAM10, -17	Kuzbanian, Kuzbanian-like, TACE	SUP-17, ADM-4	Metalloproteases, cleaving the S2-site in Notch, prerequisite for Notch signaling
PSEN1,2 (presenilin) NCSTN (nicastrin), APH1, PEN2 (presenilin enhancer)	Presenilin, nicastrin, APH1, PEN2	SEL-12, APH1, APH-2, PEN2	Proteins of the γ-secretase complex, cleaving the S3 and S4 sites in Notch, activates Notch signaling
Delta and Serrate E3-ligases (regulation of trafficking)			
MIB1,2 (mind bomb homolog) NEURL1,2 (neuralized-like)	Mind bomb 1,2 (Dmib), neuralized	Y47D3A.22	RING-finger type E3-ubiquitin ligases for Delta and Jagged/Serrate, promotes endocytosis, activation of Notch signaling
Notch E3-ligases acting in the cytoplasm (regulation of trafficking)			
ITCH NEDD4	Su(dx), Nedd4	WWP-1	HECT-type E3-ubiquitin ligase, targeting Notch for lysosomal degradation, binds PPSY motif, promotes endocytosis and lysosomal degradation, inhibition of Notch signaling
DTX1-4 (Deltex)	Deltex	No homolog identified	RING-finger type E3-ubiquitin ligase, binds ankyrin repeats, promotes Notch localization to Rab11-positive vesicles (recycling endosomes), rescue from degradation, activation of Notch signaling
Notch E3-ligase acting in the nucleus			
FBXW7	Archipelago	Sel-10	F-box protein, implicated in ubiquitylation of Notch cytosolic fragment, leading to degradation by proteasome, termination of signaling

Adapted from Fiuza and Arias (2007), Nichols et al. (2007).

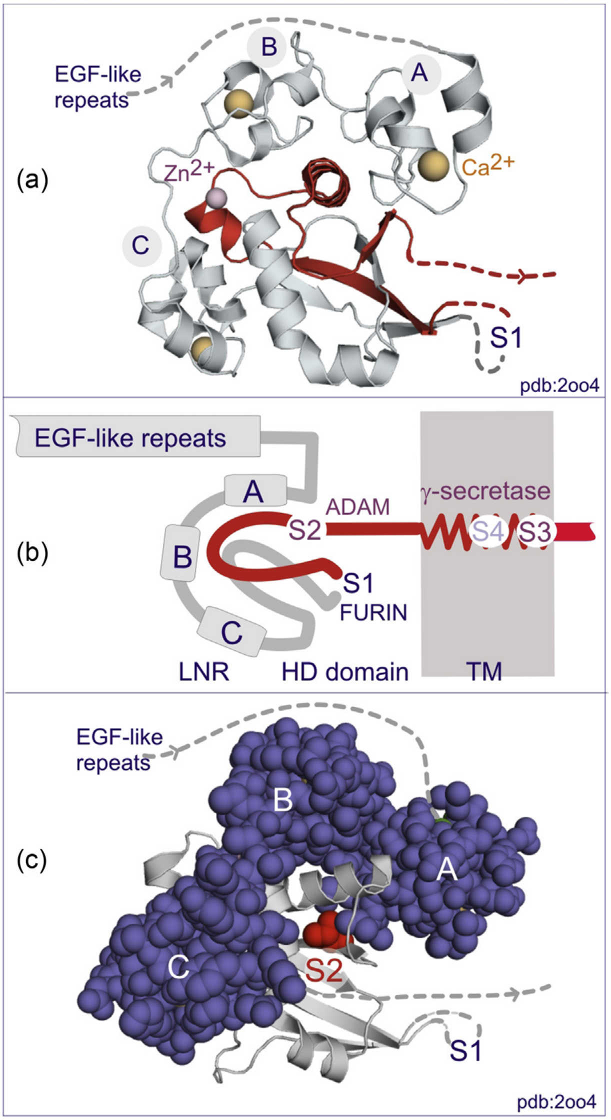

FIGURE 19-3 Structure of the Notch regulatory region. (a) Notch is cleaved in the *cis*-Golgi by furin at the S1-site and arrives at the cell surface as two subunits. The extracellular segment is indicated in gray, the transmembrane segment in red. A, B, and C indicate the three Ca^{2+}-binding Lin-12 repeats. (b) A schematic representation of the Notch protein illustrating the four cleavage sites. The S1-cleavage is ligand-independent, whereas S2, cleaved by ADAM, and S3/S4, cleaved by γ-secretase, are dependent on ligand interaction. LNR, Lin-12/Notch repeat; HD, heterodimerization domain. (c) The three Lin-12 repeats (A, B, and C) mask the S2-cleavage site. The Notch ligand renders this site accessible to the ADAM family of proteases.

Sanpodo below (see “Notch and sensory progenitor cells of *Drosophila*; the importance of endocytosis”).

Glycosylation of ligands and receptor

The fucosylation sites on the EGF-like repeats of Notch and its ligands are further modified by addition of N-acetylglucosamines (so-called glycosylation elongation). Lack of the initial fucosylation enzyme, OFUT1, results in a similar phenotype as loss of Notch (Okajima and Irvine, 2002). It may possibly also act as a chaperone in the rough endoplasmic reticulum stimulating Notch1 signaling simply by preventing protein misfolding (Okajima et al., 2005). Fringe (fng in *Drosophila* and LFNG in *Homo sapiens*), which acts in the Golgi apparatus, generally has the opposite effect, limiting signal output by O-fucose elongation of Notch1 and its ligands (Bruckner et al., 2000; Okajima et al., 2003). Regulation of OFUT1 and LFNG may contribute to the pattern of Notch activation during development.

ADAM metallopeptidase domain-containing proteins, constituting a family of 40 members plus 11 pseudogenes in the human genome, cleave the juxtamembrane region of transmembrane proteins, causing shedding of the ectodomains (for instance, liberation of TNF or EGF (return to Chapter 10, “Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases,” Figure 10-29, for the role of ADAM17 in EGF-shedding)). Among the members, ADAM10 and ADAM17 (also known as Tace in *Drosophila*) were shown to be able to cleave NOTCH1, whereas ADAM12 cleaves its ligand Delta (Tace: TNF-alpha converting enzyme).

ACTIVATION OF NOTCH1

Cleavage of the receptor

Signaling through NOTCH1 occurs only between cells that are in direct contact with each other (Heitzler and Simpson, 1991). Activation entails proteolytic processing at two sites. First, ligand binding induces cleavage at the S2-site by members of the ADAM family of metalloproteases. This sets the stage for a second cut by the γ-secretase complex, which cleaves the S3-site in the transmembrane region (Schroeter et al., 1998; Mumm et al., 2000). There is a further cut by γ-secretase at S4, right in the middle of the transmembrane domain but the functional significance of the resulting NOTCH1 fragment has not been investigated. We limit our description to

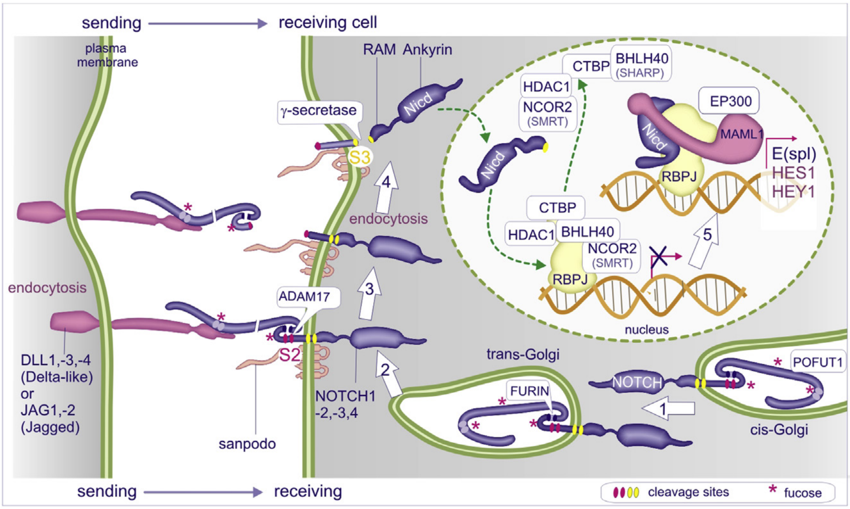

FIGURE 19-4 Activation of the Notch pathway. NOTCH1 appears at the plasma membrane in a glycosylated (1) and cleaved (2) form. It binds its partner Sanpodo. Binding of ligand (Delta or Jagged) exposes the S2-site, leading to cleavage by ADAM17 (3). This renders NOTCH1 susceptible for cleavage at S3 by γ-secretase (4). Endocytosis of the receptor seems a prerequisite for this event. The intracellular segment, Nicd, translocates to the nucleus where it binds RBPJ and causes the recruitment of mastermind (MAML1). This leads to a loss of RBPJ-associated repressors, which are replaced by MAML1-associated activators of transcription (5). Important target genes are members of the Enhancer of Split family (E(spl)), in *Drosophila*, of which HES and HEY are the equivalent families in mammals.

the signaling role of the S3 NOTCH1-intracellular domain (NOTCH1-icd or Nicd) (Figure 19-4).

What exactly renders NOTCH1 sensitive to proteolytic cleavage following ligand binding remains unclear, but the removal of the inhibitory Lin-repeats, which mask the S2-cleavage site, seems to be a prerequisite. Why the truncated version then becomes susceptible to the action of γ-secretase also remains enigmatic. Binding of DELTA or JAGGED and their subsequent endocytosis may provide the traction force that exposes the S2-cleavage site in NOTCH (Tiyanont et al., 2011). Curiously, the number of EGF-repeats seems to determine the efficiency of proteolysis. Long chains are less efficient and, on the contrary, truncated versions, as occur in the human oncogenes Tan1 (NOTCH1) and Int3 (NOTCH4), are constitutively active receptors (Radtke and Raj, 2003). Both ADAM and γ-secretase are subject to regulation by posttranslational modifications that affect their activity. In which subcellular compartment γ-secretase cuts NOTCH1 remains to be resolved. Although clearly evident in early endosomal vesicles, it does not necessarily follow that this is where it acts (Black et al., 1997).

γ-Secretase is a transmembrane multiprotein protease complex consisting of four different proteins: the aspartyl protease presenilin (PSEN1 or PSEN2), surrounded by nicastrin (NCSTN), APH1, and PEN2 (presenilin enhancer). The latter induces endoproteolysis of the catalytic subunit, required for activity. Loss of function of presenilin correlates with accumulation of plaques in Alzheimer's patients (extracellular deposition of amyloid-β42 polypeptide-aggregates) (Shen and Kelleher, 2007). It constitutes a focus of interest in terms of pharmacological intervention (e.g., see Wolfe, 2006), in the hope that it may be possible to define new targets in cancers in which mutations of NOTCH contribute to the pathology (such as T-cell acute lymphoblastic leukemia) or in which NOTCH acts to prevent progenitor cell differentiation such as in intestinal adenomas.

Regulation of transcription by the NOTCH1-intracellular domain

The untethered intracellular segment of NOTCH1 (Nicd) migrates into the nucleus there to compete for binding with a number of repressor proteins (such as HDAC, NCOR2, CTBP, BHLH40) for the transcription factor RBPJ (formerly CBF1 or CSL). The interaction with RBPJ first involves the RAM region, which binds the β trefoil domain of RBPJ, and then the ankyrin repeats, which bind the C-terminal REL-homology region (Figure 19-5) (Kovall and Henderickson, 2004; Fortini and Artavanis-Tsakonas, 1994; Struhl and Adachi, 1998). The ankyrin repeats also interact with SKIP, a protein that also makes contact with RBPJ (Zhou et al., 2000). Together, Nicd and RBPJ create a binding groove that accommodates the helical structure of mastermind-like MAML1 which creates a platform for attachment of further components such as the nucleosome acetylation factors CREBBP or EP300. It is also involved in the recruitment the cell cycle kinase CDK8 (itself associated with the mediator-complex) (Taatjes, 2010) (return to Chapter 7 "Bringing the Signal in the Nucleus: Regulation of Gene Expression," section "Transcription and transcription factors" and Chapter 17, "TGFβ and Signaling through Receptor Serine/Threonine Kinases," section "Activation of gene expression") (Fryer et al., 2002, 2004). Numerous of these complexes form and they are readily visible, by immunocytochemical staining of EP300, as distinct nuclear foci (Figure 19-5).

Purified mammalian RBPJ proteins bind as monomers to DNA at a consensus sequence of (C/A/T)(G/A)TG(G/A/T)GAA (Tun et al., 1994). However, certain enhancer regions, such as those of the genes that code for HES1 and HES4, contain a paired site, where the first response element contains a consensus DNA sequence and the second, rough 15 base pairs

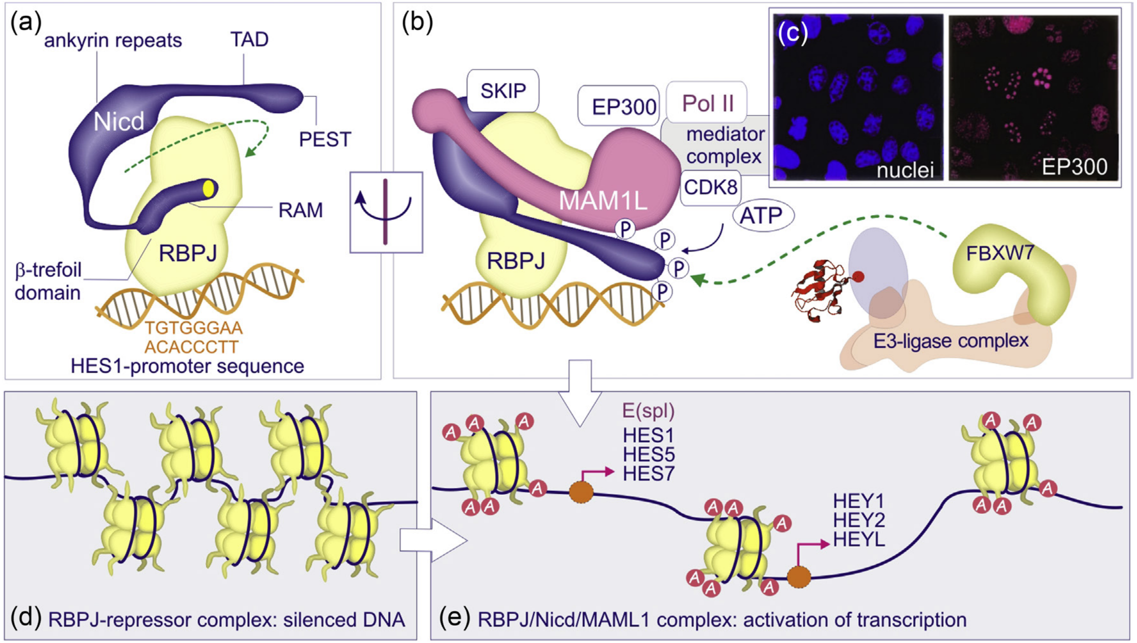

FIGURE 19-5 The NOTCH1 transcriptional complex. (a) The intracellular NOTCH1 segment, Nicd, first binds the β-trefoil domain of RBPJ and then folds over to fix with its ankyrin repeats to the C-terminal. (b) This creates a binding site for Mastermind (MAML1) and a tripartite complex is formed. The C-terminal domain of MAML1 recruits numerous proteins that cause acetylation of the DNA so rendering it amenable for transcription. Among those recruited is CDK8, which phosphorylates Nicd at both the TAD- and PEST regions. The phosphorylated PEST is recognized by the receptor-subunit of a nuclear E3-ligase complex resulting in polyubiquitylation of Nicd, followed by proteasome-mediated destruction. (c) Numerous transcriptional complexes thus formed are readily visible by immunostaining of EP300 *(image from Fryer et al. (2002))*. (d) and (e) Transition from an inaccessible to accessible DNA by removal of repressors and recruitment of transcriptional activators.

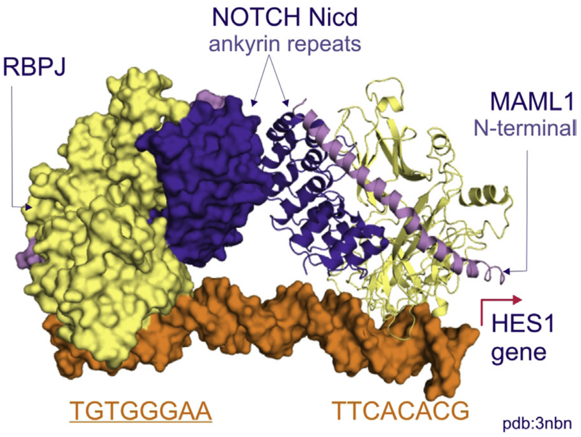

FIGURE 19-6 Head-to-head binding of NOTCH intracellular domain, MAML1 and RBPJ to a paired response element of the human HES1 promoter. The left protein complex binds to the consensus sequence (underlined). The second complex binds in the inverse direction to a DNA sequence that functions as a binding site only when the two ankyrin motifs of NOTCH-Nicd interact (cooperative assembly). The complex on the left is shown in surface representation and the complex at the right in cartoon. Note the long helix of the N-terminal segment of MAML1 that aligns the binding groove at the interface of Nicd and RBPJ.

away, contain a sequence that by itself only binds RBPJ weakly but will bind as a consequence of protein–protein interaction between the ankyrin repeats of two Nicd segments (cooperative assembly) (structure shown in Figure 19-6) (Arnett et al., 2010).

The Notch transactivation process generally resembles the mechanism of induction of transcription by the WNT/β-catenin pathway. Thus, the NOTCH and WNT target genes are normally repressed by the association of corepressors with RBPJ or with TCF7L1. The Notch transactivation mechanism, however, differs slightly in that after translocation the Nicd requires both RBPJ and MAML1 in order to initiate transcription on chromatin templates in vitro, whereas β-catenin requires only TCF7 (or LEF1) (Fryer et al., 2002; Tutter et al., 2001). Of note, RBPJ may also repress basal transcription through direct binding to, and inhibition of TFIIA and TFIID (components of the transcriptional promoter complex) (Olave et al., 1998).

Effector genes of NOTCH signaling

In *Drosophila*, the Nicd-induced transcriptional complex leads to the expression of genes that are members of the E(spl) (enhancer of split genes, constituting a complex of at least seven tandem genes) (Lecourtois and Schweisguth, 1995; Fischer and Gessler, 2007). The mammalian counterparts are two closely related classes of genes, HES and HEY (7 HES (1–7) and 3 HEY (1, 2 and L)). These are the so-called Notch-effectors and they belong to a large family of small transcription factors characterized by a helix-loop-helix (HLH) motif (Massari and Murre, 2000). Well-known members of this family of DNA-binding transcription factors are MYOD (differentiation of muscle tissue) and MYC (oncogene, regulation of expression of components of ribosomes).

> Split is one of the nonlethal alleles of the Notch locus. It is a partial loss-of-function mutant that affects formation of the bristles as well as the eyes. The macrochaetae, particularly, the dorsocentrals and the scutellars on the mesonotum of the thorax, appear split, doubled, or absent, as do some of the microchaetae. The eyes are of rough, of unvarying texture, and are smaller than the wild type (Stern and Tokunaga, 1968).
>
> Enhancer of split exaggerates for the worse the effects of split. In genetic terms, Split and Enhancer of split are said to interact.

The N-terminal DNA-binding basic domain of the HLH proteins is contiguous with one of two α-helices separated by a loop (HLH) that serves as a dimerization domain and as a platform for additional protein interactions (Figure 19-7). This is followed by two additional α-helical stretches, the Orange domain, which also serves as an interface for protein interactions, including the formation of homo- and heterodimers of HLH proteins. The Orange domain also acts as a transcriptional repressor. In HES proteins, a highly conserved carboxyterminal tetrapeptide motif WRPW recruits the corepressors of the TLE family, of which Groucho (*Drosophila*) is the most familiar. The HEY proteins lack this motif and cannot bind TLE but their bHLH motif interacts with yet another histone deacetylase, SIRT1. The corepressors and associated histone deacetylases render the chromatin inaccessible to RNA polymerase and other regulatory transcription factors and thus silence gene expression. In some cases, HES proteins bind CREBBP and act as transcriptional activators. Apart from binding DNA and recruiting repressors or activators of transcription, bHLH proteins have other means of interfering with transcription. For instance, they may sequester other DNA-binding proteins away from the DNA or they may bind other transcription factors thus bringing gene silencing repressors to promoter regions where they themselves do not bind. For more information about HES and HEY proteins, see Fischer and Gessler (2007).

The response to NOTCH1 is not fixed by expression of a set of transcription factors that endow certain phenotypes but rather it acts as a switch with the outcome of its action being determined by the context in which it operates (cell type, developmental stage, other first messengers). The situation is similar to what we described for Wnt (discussed in Chapter 12, “WNT Signaling and the Regulation of Cell Adhesion and Differentiation”). In this way, it can be used in cell fate decisions at many stages

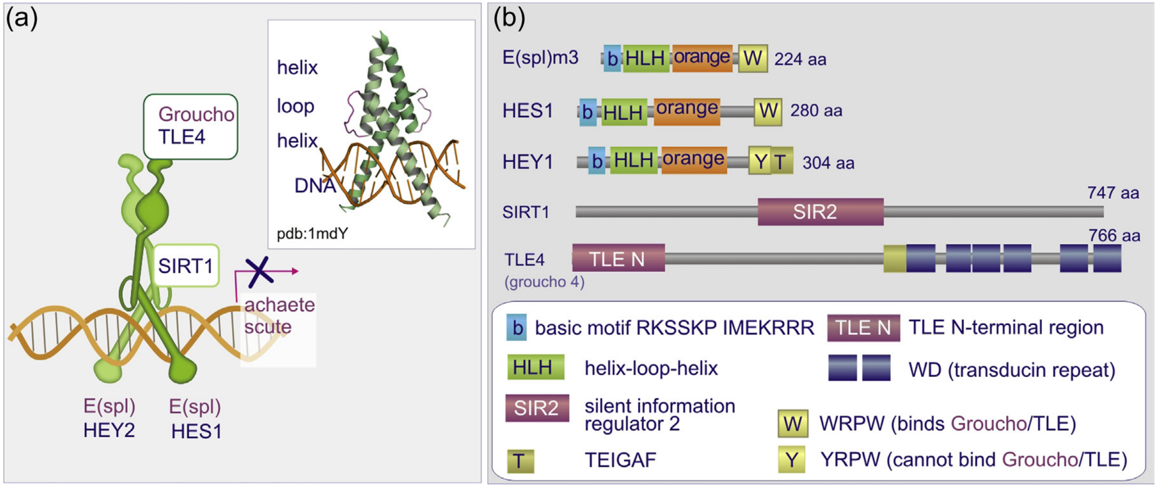

FIGURE 19-7 Molecular structure and domain architecture of NOTCH1-effectors. (a) NOTCH1 target genes are members of the basic helix-loop-helix (bHLH) class of transcription factors (the insert represents the structure of MYOD). These bind DNA as homo- or heterodimers. In *Drosophila*, Notch induces expression of E(spl), which in turn binds Groucho and leads to suppression of expression of achaete and scute, thereby, blocking the neural differentiation pathway. In mammalian cells, NOTCH1 induces HES and HEY, members of the same bHLH family. The C-terminal WRPW motif of HES1 interacts with TLE4, whereas HEY2 binds SIRT1 with its HLH motif. Together they repress gene expression. (b) Domain architecture of NOTCH1 effector-proteins. SIRT1 and TLE4 are histone deacetylases.

of the developing embryo. In *Drosophila*, Notch plays an important role in determining the choice between epidermal and neural development; its activation prevents neurogenesis. More details on this later in section "Notch and sensory progenitor cells of *Drosophila*; the importance of endocytosis".

Gene cassettes and the Enhancer of Split complex, E[Spl]: In *Drosophila*, genes are often arranged according to their function on the chromosome. A group of genes, a cassette, having related functions and sharing a location on the chromosome signifies a so-called gene complex. The E[spl] complex comprises eight genes spread over 50 kilobases on the third chromosome. Other examples of *Drosophila* gene complexes include the Antennapedia complex (ANTP complex), the bithorax complex (BX complex), and the achaete–scute complex (AS complex). The hallmark of all these complexes is that within them, the genes are evolutionarily related and jointly regulated. Genes of the E(spl) and the AS complex regulate neurogenesis and related differentiation pathways. The transcription factors encoded by the AS complex generally activate other genes and, conversely, products of E(spl) are generally repressive. Achaete/scute encoded proteins are proneural, they initiate neurogenesis, whereas E(spl)-coded proteins prevent neurogenesis. The E(spl) complex contains three additional Notch-responsive, non-bHLH genes: of these, *m*4 and *mα* are structurally related, while *m*2 encodes a novel protein. The genes of E(spl) complex are for the most part, redundant, with only the defining gene of the complex, *Enhancer of Split* yielding a noticeable phenotype when mutated.

Biological functions in humans

In mammals, HES and HEY proteins have a role in the development of the nervous system, sensory organs (eye, inner ear); pancreas and endocrine cells, as well as lymphocytes. Hey genes are critical in the cardiovascular system, their dysfunction causing congenital defects, impairments of angiogenic remodeling, and a lack of arterial differentiation. As we will discuss later, in intestinal crypts of mice, HES1 represses the expression of ATOH1 (formerly Math1), an essential transcription factor for the differentiation of uncommitted progenitor cells (or transit amplifying cells) toward secretory cells (Paneth or goblet cell) (Fischer and Gessler, 2007; van de Es et al., 2010).

DESTRUCTION OF THE NOTCH1-INTRACELLULAR DOMAIN, NICD

The nuclear content of Nicd is vanishingly small due to rapid degradation. In fact, the assembly of a transcriptional active protein complex is paralleled by an almost immediate phosphorylation of Nicd and subsequent recognition by a nuclear E3-ubiquitin ligase so that it signals its own demise. Recruitment of CDK8 leads to phosphorylation of Nicd in the TAD- and PEST motif (Figure 19-5). The phosphorylated protein is recognized by the receptor-subunit FBXW7 (a WD40 F-box receptor protein) of a nuclear SCF-type E3-ubiquitin ligase complex. The polyubiquitylated Nicd is then recognized and destroyed by the 26S proteasome (Lai, 2002; Fryer et al., 2004). If signaling is to be maintained, a continuous supply of Nicd must be provided through ligand-mediated cleavage of the intact membrane protein.

The importance of a continuous supply of ligand is nicely illustrated by the observation that severe suppression of Delta expression in the satellite cells of aging livers leads to impaired regenerative capacity; due to a failure of Notch signaling, the cells can no longer escape terminal differentiation (Conboy et al., 2005). Conversely, loss of the PEST motif renders the protein very stable and this contributes to the development of acute lymphoblastic leukemia (an excess of undifferentiated cells) (see Figure 19-18) (Weng et al., 2004).

BOTH RECEPTOR AND LIGAND TRAFFICKING ARE ESSENTIAL FOR NOTCH SIGNALING

One might expect that signal-sending cells would exhibit high levels of ligands on their surface so as to activate NOTCH1 on adjacent receiving cells. Curiously, in *Drosophila,* most of Delta is confined to intracellular vesicles and the membrane pool is constantly being removed. Moreover, only ubiquitinylated ligands, linked to clathrin-associated sorting proteins, are competent to activate NOTCH1.

Compelling evidence for a role of trafficking came from the finding that "shibire" (shi), a temperature-sensitive mutant of dynamin causes a Notch-like phenotype in *Drosophila*. At the restrictive temperature (29 °C), shibire mutants develop an excess of nervous system at the expense of ventral epidermis (Poodry et al., 1971). Since Notch signaling is often required to prevent the default neurogenic phenotype (thus to obtain an epidermal cell instead), it follows that loss of function of dynamin prevents effective Notch signaling (even though all the related components, receptors and ligands etc., are normally expressed). Shibire codes for dynamin is a GTP-binding protein necessary for the pinching-off (fission) of endocytotic vesicles from the plasma membrane (Chen et al., 1991). Cells that express shibire still form clathrin-coated pits but fail to internalize these vesicles. This correlation prompted the question of whether a block in endocytosis could account for the interruption in the communication necessary for normal epidermal and neural cell differentiation? The answer is yes, trafficking of both ligands and receptor affect signaling though the full picture still remains rather fuzzy.

Trafficking of ligand (Delta, Serrate)

Further important evidence for the role of endocytosis in the regulation of Notch signaling came from genetic screens in *Drosophila* and zebrafish. These revealed three key regulators: lap (epsin), neur, and mib1 (Table 19-1). Lap is a long almost linear protein with a globular epsin-N-terminal homology domain (ENHT) that forms a binding pocket for phosphatidylinositol lipids. It acts as an adaptor for endocytosis, coupling polyubiquitylated protein to α-adaptin (AP-2) and it plays a role in bringing about membrane curvature (Ford et al., 2002; Hawryluk et al., 2006). Neur (neuralized) is a RING-type zinc-finger-containing protein that acts as an E3-ubiquitin ligase, whereas mib1 (mind bomb) is a HECT-domain E3-ubiquitin ligase. They ubiquitinate Delta and Serrate (Le Borgne et al., 2005). Loss of Neur in *Drosophila* and *Xenopus laevis* and of mib1 in zebrafish results in neurogenic phenotypes resembling those of Notch mutants (Bray, 2006). Two schemes have been proposed to explain why endocytosis is necessary for proper signaling. The first postulates that uptake of ligand, when bound to the Notch receptor, pulls away the three Lin-repeats that normally mask the S2-cleavage site. This "pulling" renders S2 accessible to ADAM17 (Tace in *Drosophila*) (see Figures 19-3 and 19-7) (Tiyanont et al., 2011). Thus, ligand cells defective in endocytosis bind and cluster Notch, yet fail to activate it. The other idea proposes that ligand uptake leads to glycosylation of the EGF-like repeats in the early endosome compartment. This recycling pathway not only renders the ligand competent for receptor binding but it may also deliver the ligand to the correct membrane domain in a "clustered" manner (Figure 19-7). It is not excluded, as a third possibility, that trafficking of Delta and Serrate, after binding to Notch, is required for signaling within the ligand-presenting sending cell, in a similar way as for Notch in the ligand-receiving cell (Le Borgne et al., 2005). Recycling of ligand is certainly crucial for cell fate termination in sensory organ precursor (SOP) cells in *Drosophila* (see below) but not an essential requirement in other contexts in which Notch operates.

Epsin (EPN1) is described as a "natively disordered" polypeptide, meaning, that apart from the N-terminal region, it has few structural features (Lobley et al., 2007). It contains three ubiquitin-interacting motifs which, however, bind weakly to ubiquitin. Tight binding therefore requires several interactions and this may explain why polyubiquitylated proteins are favored. It connects to phosphatidylinositol-4,5-bisphosphate and it carries several AP-2 binding motifs that bind α-adaptin. Epsin also interacts with RhoGAP, suggesting that it has a role in actin dynamics, possibly explaining why it is an important component of the endocytosis machinery (Hawryluk et al., 2006; Aguilar et al., 2006; Owen et al., 2004).

Trafficking of receptor (NOTCH)

Ubiquitylation, and the subsequent endocytosis, of NOTCH has both stimulatory and inhibitory consequences. The inhibitory aspect is rather straightforward; it concerns removal of the unoccupied receptor from the plasma membrane, thereby, preventing interaction with ligand. Two ubiquitin E3-ligases stand out in the regulation of endocytosis; NEDD4 and ITCH, both HECT-type E3-ubiquitin ligases (Figures 19-8 and 19-9, and Table 19-1). For more information about E3-ubiquitin ligases, return to Chapter 13, "Activation of the Innate Immune System; the Toll-Like Receptor and Signaling through Ubiquitinylation," Figure 13-16. Ubiquitylation conducts NOTCH into an early endosome, identified by the colocalization of the monomeric GTPase RAB5, and, if no further modifications occur, this is followed by its destruction in the lysosomal compartment (recognized by association of RAB7).

Itch is a gene discovered in mice that do not stop scratching their skin. This is due to aberrant inflammatory responses in several organs, including the skin (Perry et al., 1998).

Why NOTCH is stimulated by ubiquitylation and endocytosis is less clear. One possibility, based on the finding that presenilin (PSEN1), the catalytic component of γ-secretase, predominantly resides in the early and late endosomes, is that this might facilitate cleavage of the S2-site by γ-secretase (Nichols et al., 2007) (Figure 19-9). Another possibility is that a second round of ubiquitylation, in the early endosomal compartment, rescues Notch from lysosomal destruction, so opening the way for an alternative, noncanonical, signaling pathway. This second round of ubiquitylation occurs through Deltex-1 (DTX1, a RING-finger type E3-ligase), which binds the ankyrin repeat region (Matsuno et al., 1995). DTX1-mediated ubiquitylation directs NOTCH toward a recycling endosome, identified by the colocalization of the small GTPase RAB11. From here, Notch either returns to the membrane, possibly modified, and contributes to ligand-mediated signaling or it remains in the cell and signals, by an as yet poorly characterized pathway, in a ligand-independent manner (Ramain et al., 2001; Hori et al., 2004). Of note, the E3-ubiquitin ligase CBL, which regulates EGF receptor uptake (see Chapter 10, "Regulation of Cell Proliferation by Receptor Tyrosine Protein Kinases," Figure 10-26) is also associated with NOTCH receptor uptake and could also play a role in its trafficking.

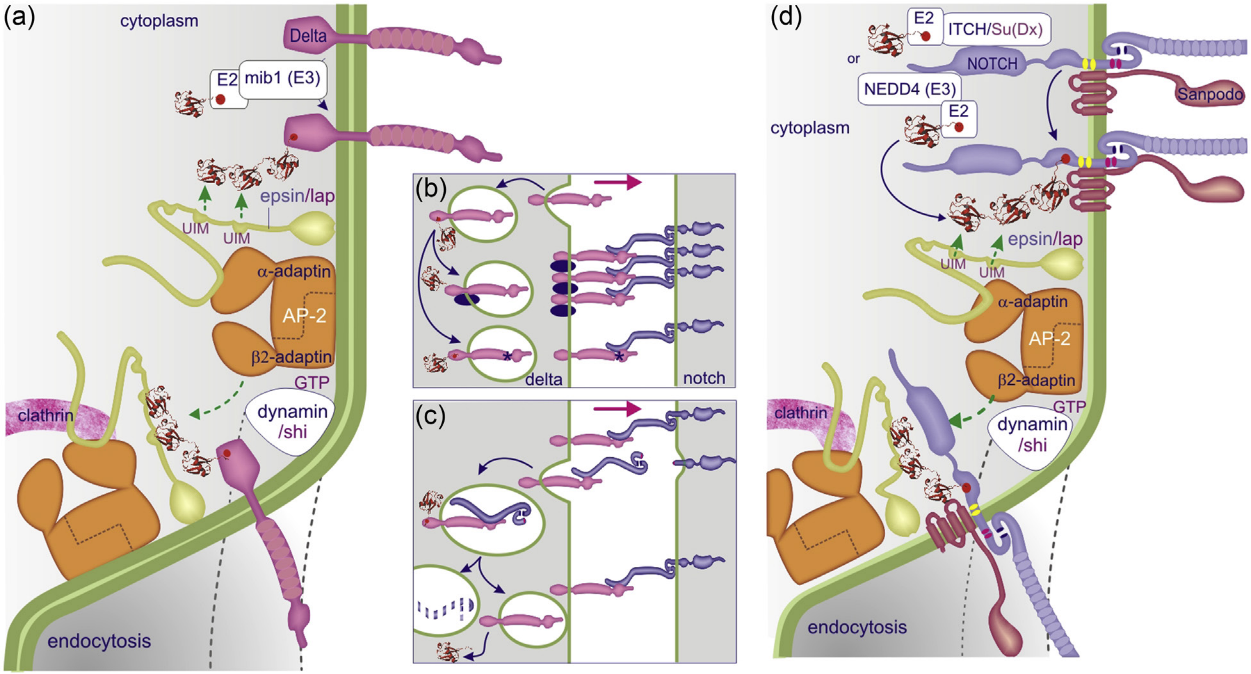

FIGURE 19-8 Endocytosis of the Delta ligand and of the Notch receptor. (a) Delta is ubiquitinylated by the E3-ligase mib1 (or neur). The polyubiquitin-chain is recognized by the ubiquitin-interacting motif (UIM) of Lap (epsin), a membrane-associated protein, which links Delta to the adaptor complex AP-2, leading to its endocytosis with the help of the coat protein clathrin. (b) Endocytosis may facilitate Notch signaling in a number of ways. It may be reexpressed at the membrane with important cofactors and in a clustered manner which may enhance Notch binding and activation. It may also undergo further glycosylation (symbolized as asterisk). (c) Endocytosis may facilitate unmasking of the S2-cleavage site by withdrawing the Lin-12 repeats. Finally endocytosis may be needed for the effective recycling of ligand after removal of the extracellular Notch segment. (d) Endocytosis of the Notch receptor. Notch, polyubiquitinylated by ITCH (Su (Dx) in *Drosophila*) or NEDD4. The ubiquitin-chain is recognized by Lap and this connects Notch with the adaptor protein complex AP-2. Endocytosis occurs with the help of the coat protein clathrin.

RAB GTPases: These are members of the RAS superfamily of monomeric GTPases involved in intracellular vesicle transport. They regulate the assembly, on the surface of emerging transport vesicles, of: (a) coat proteins, necessary for cargo selection and vesicle budding; (b) microtubule-binding proteins, necessary for transport; and (c) SNARE and docking proteins, necessary for fusion of the released vesicle with other subcellular membrane compartments.

AP-2: A complex comprising four subunits (two large α- and β1-adaptin and two small μ1- and σ1-adaptin), of which μ-adaptin has the role of cargo receptor. Human gene names for the complex are AP2A1, AP2B1, AP2M1, and AP2S1. The "ear" domain of α-adaptin interacts with accessory proteins, such as epsin or numb, and coat proteins, such as clathrin.

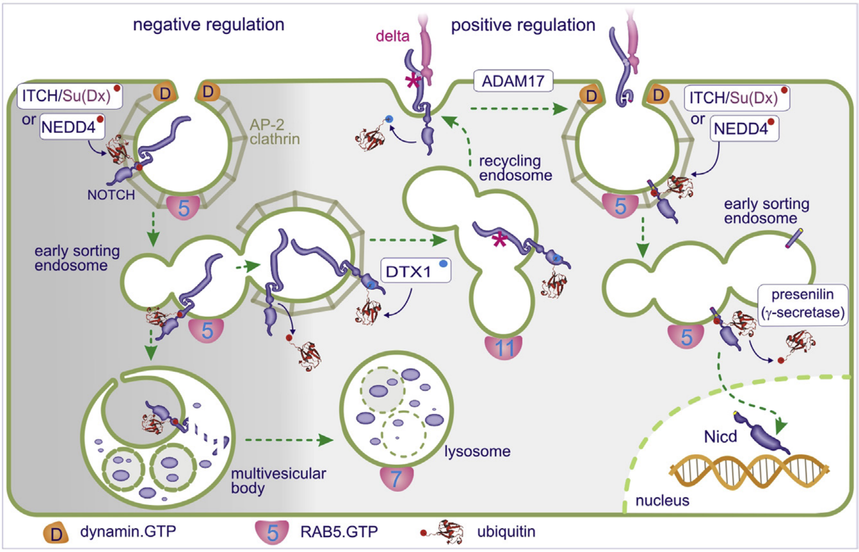

FIGURE 19-9 Intracellular trafficking of the Notch receptor. Intracellular trafficking of the NOTCH receptor has negative and positive effects on its signaling capacity. Receptor endocytosis and conduct toward early sorting endosomes (marked by RAB5) normally results in partial destruction in multivesicular bodies and then total degradation in the lysosome (marked by RAB7). This effectively removes the receptor from the surface. Alternatively, endocytosis may positively regulate signaling. First, by protecting the receptor from destruction and conducting it to recycling endosomes (marked by RAB11) assisted by DTX1-mediated ubiquitinylation, which provides a novel sorting signal. From here, the receptor returns to the membrane, perhaps in a modified version (indicated by an asterisk) or at a membrane domain more favorable for ligand binding. Second, endocytosis also favors proteolysis of the S2-site by bringing the receptor to the compartment that harbors γ-secretase (early and late endosomes). Dynamin (D) and RAB5, -7, and -11 are marked in blue numbers. Red spots indicate ITCH or NEDD4, Blue spots indicate DTX1 (Deltex).

NOTCH IN *DROSOPHILA* DEVELOPMENT

Introductory note

The embryonic life of a fruit fly is brief, about a day. On hatching, as a first instar larva, it then starts a second life in three stages, all dedicated to eating. It grows into a second instar and finally, after a copious intake of food, achieves the third instar larval state. All this takes about 5 days after which the larva molts, crawls out of its food source to emerge as a pupa. It then remains this way for another 5 days at the end of which, we have an adult fly.

Here we discuss the formation of cuticular patterns, the cellular machinery that generates them, and the genetic circuitry that organizes the machinery. All the events described start at the pupal stage, where the imaginal discs, already visible in 3rd instar larvae, expand and develop into the body parts of the adult fly. This process is known as metamorphosis (Figure 19-11). During this process most of the larval structures are destroyed through apoptosis and replaced by tissue derived from the discs, giving rise to thorax, wings, legs, antennae, and genitalia (though not the digestive system). Full-grown larvae are roughly 6 mm, whereas the adult

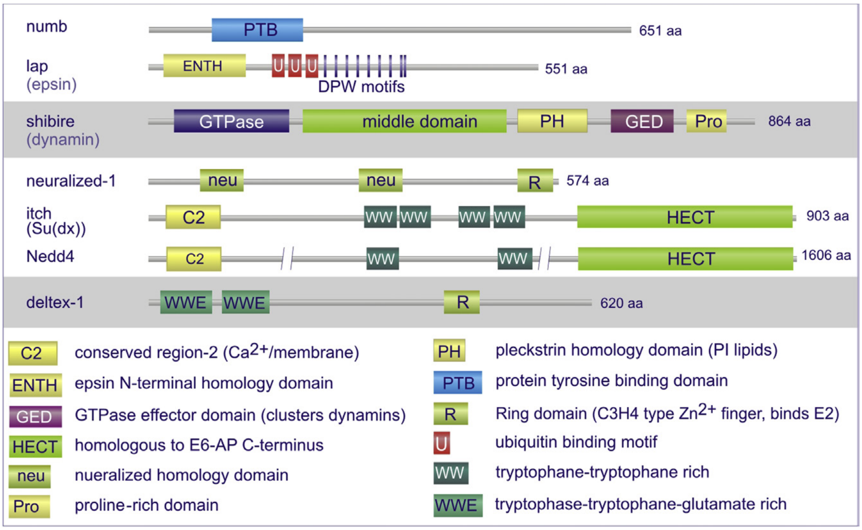
numb
PTB
651 aa
lap
(epsin)
ENTH
U U U
DPW motifs
551 aa
shibire
(dynamin)
GTPase
middle domain
PH
GED
Pro
864 aa
neuralized-1
neu
neu
R
574 aa
itch
(Su(dx))
C2
WW WW
WW WW
HECT
903 aa
Nedd4
C2
WW
WW
HECT
1606 aa
deltex-1
WWE WWE
R
620 aa
C2 conserved region-2 (Ca2+/membrane)
ENTH epsin N-terminal homology domain
GED GTPase effector domain (clusters dynamins)
HECT homologous to E6-AP C-terminus
neu nueralized homology domain
Pro proline-rich domain
PH pleckstrin homology domain (PI lipids)
PTB protein tyrosine binding domain
R Ring domain (C3H4 type Zn2+ finger, binds E2)
U ubiquitin binding motif
WW tryptophane-tryptophane rich
WWE tryptophase-tryptophane-glutamate rich

FIGURE 19-10 Domain architecture of proteins that affect Notch signaling. Numb and lab (epsin) link Sanpodo and Notch with the endocytotic machinery (AP-2 and clathrin), whereas shibire (dynamin) is required for fission of the endocytotic vesicle. Neuralized (neur), Itch, Nedd4, and Deltex all are E3-ubiquitin ligases that ubiquitylate the Notch receptor and its ligands, thereby, providing sorting signals for the endocytotic machinery. A homolog of Sanpodo (*Drosophila*, gene name, spdo) has not been discerned in humans but the role is taken by numb-like (NUMBL) which associates with both NOTCH1 and EPS15.

fruit fly is only half that size. When the adults emerge from the pupae, they are fully formed. They become fertile after about 10h, copulate, the females lay eggs, and the cycle, of roughly 12–14days, begins again.

> Like other holometabolous insects, flies live two lives, first as a grub, then as a flying adult. During metamorphosis, 19 "imaginal discs" erupt from inside the maggot and are quilted together to form most of the adult skin. The gold-colored cuticle secreted by that skin is exquisitely ornate. The head is embossed with hundreds of domes that focus light onto bundles of photoreceptors, the thorax is sculpted into dozens of jointed parts that form a contraption for walking and flying, and the abdominal wall (built from non-disc tissue) is pleated into an expansible chamber for digestion and reproduction. Nearly everywhere, the body surface sprouts bristles whose patterns can be as orderly as soldiers on parade. ***Quoted from Held (2005).***

Macrochaetes and microchaetes. Precursors of the macrochaetes appear early in the larval period. Due to the long period of intervening growth before metamorphosis, these bristles are large and widely spaced. Precursors of the microchaetes appear later, during the pupal stage and as a result they are smaller and more closely spaced. Macrochaetes are longer, thicker, and stouter than microchaetes. They are arranged into a stereotyped array. Each macrochaete has a specific axonal projection pattern in the thoracic ganglion that depends upon the position at which the bristle precursor first emerges (Ghysen, 1980). The notal macrochaetes of *Drosophila* have directional sensitivity, their neurons responding to movements of the shaft in a preferred axis. Although they display some regional specificity, microchaetes are variable in number and position and do not appear to have individually defined functions (Usui-Ishihara and Simpson, 2005).

Development of mechanoreceptors on thorax and wing

The pair of large wing discs produces both wings and the dorsal site of the thorax (notum) (Figures 19-11 and 19-12). We focus on the proneural clusters, small patches of cells within these discs. They are detected by expression of neurogenic markers, such as the genes achaete, scute, and daughterless (Figure 19-11(b)), the gene products of which, confer epidermal cells the ability to become sensory organ precursor cells (SOPs). The cells surrounding the proneural clusters express high levels of *hairy*, a bHLH protein that suppresses the neurogenic genes. Hairy belongs to same family as the previously described E(spl), HES and HEY genes,

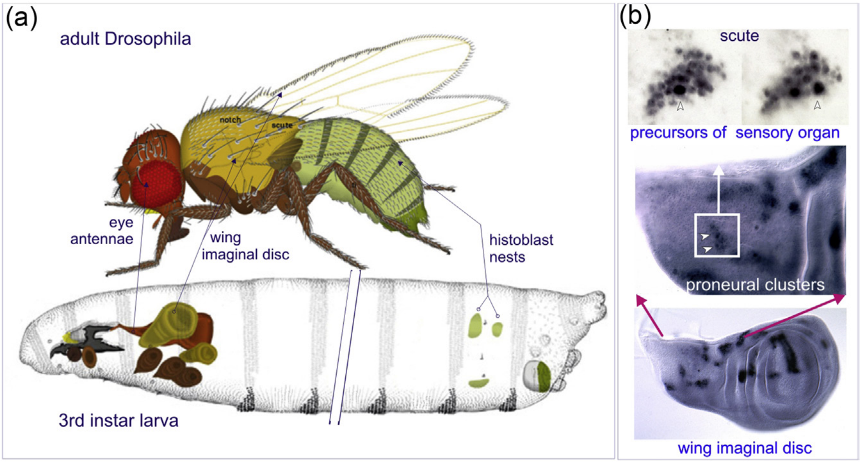

FIGURE 19-11 Imaginal discs of a 3rd instar larva and the corresponding body parts of the adult fly. (a) The imaginal discs harbor cells that form, during the process of metamorphosis, the different body parts of the adult fly. Here, only the eye- and the wing imaginal discs are indicated. The abdomen arises from histoblast nests. Note that the larva is much bigger than the adult, most of the cells outside the imaginal discs having been removed by apoptosis. *(Image adapted with permission from V. Hartenstein, http://flybase.bio.indiana.edu/.)* (b) The imaginal wing disc contains clusters of cells, proneural clusters, that express the neurogenic gene achaete. These clusters later form the mechanosensory organs. In each selected cluster, just one cell that expresses a particularly high level of achaete retains the neural fate. The others develop as epidermal cells. *Immunochemical staining of achaete adapted from Cubas et al. (1991).*

but it is regulated differently. These *achaete*-expressing proneural clusters will give rise to the bristle-bearing sensory organs (mechanical sensors) that adorn the dorsal side of the thorax and the edges of the wings (also known as macro- and microchaetes). Mechanoreceptors are also abundantly present on the abdomen, legs, and eyes, but these arise from other imaginal discs. (These structures should not be confused with the wing hairs that are much smaller and arise from epidermal cells, not neuronal cells.) Within the proneural clusters, only a single cell, the SOP cell, has the privilege of developing the organ, providing the neuronal glia, sheath, socket, and bristle cell. The other cells are ruled out through lateral inhibition, and make their contribution in the formation of the epidermis (which produces the cuticular exoskeleton) (Figure 19-12).

Comprehensive information regarding the bristle-containing sensory organs, can be found in the excellent multimedia resources of Lewis Held on the Web site of the Society of Developmental Biology: http://www.sdbonline.org/fly/lewheld/00idheld.htm, section “the bristle.”

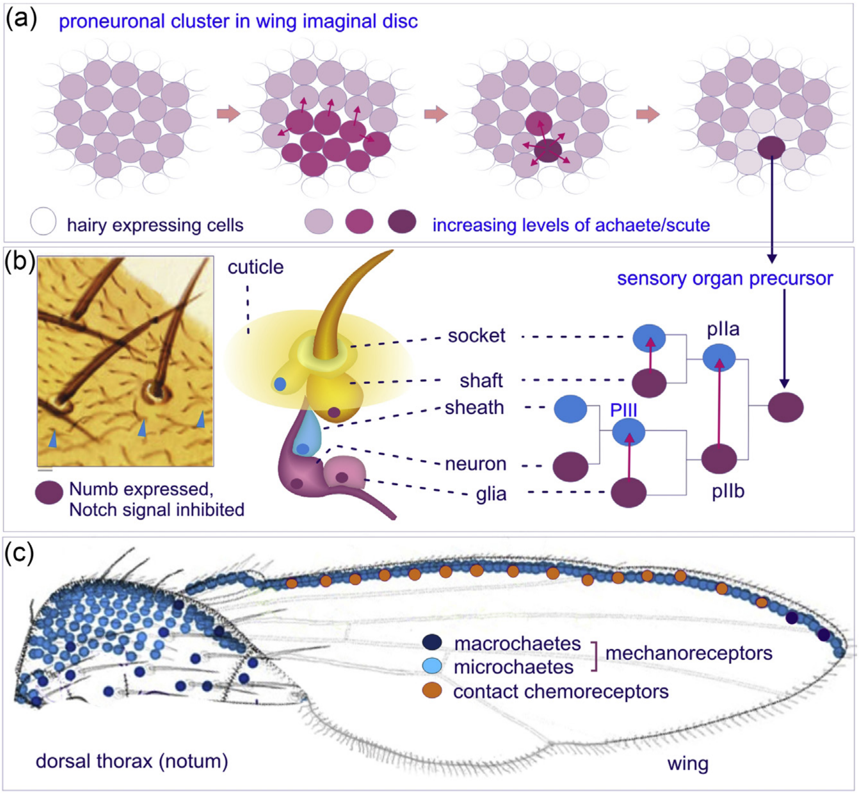

FIGURE 19-12 Sensory organ development from proneural clusters in the wing imaginal disc. (a) Proneural clusters in the wing imaginal disc express (among others) the neurogenic genes achaete and scute. Through lateral inhibition, a progressively smaller group of cells maintains expression of these genes, whereas others loose them and take on an epidermal fate. This process eventually singles out just one cell, designated the sensory organ precursor (SOP) cell. (b) The SOP cells divide and again through lateral inhibition, just a single cell maintains the neural phenotype (pIIb), the other (pIIa) losing it. Notch then plays a further role in determining cell lineage (shaft vs socket, sheath/neuron vs glia). The process represents a series of binary switches in which Notch determines the outcome. What we describe here applies for the sensory organs with the small bristles but the same principle applies for development of the organs having the large bristle. (c) Schematic presentation of the localization of sensory organs bearing small bristles (microchaetes) and large bristles (macrochaetes) on the thorax and wings of *Drosophila*. *Image adapted with permission from V. Hartenstein*, http://flybase.bio.indiana.edu/.

Mutations that affect the number or the pattern of sensory organs are thus readily observed by the change in the number or the pattern of the sensory bristles. For example, loss-of-function mutations in the gene *achaete*, which codes for an activating bHLH transcription factor that drives differentiation of neurons, leads to flies having a sparse covering of hairs.

Conversely, loss-of-function mutation of the gene "suppressor of hair" results in flies having an excess of sensory bristles (ectopic production).

Notch and the development of the bristle-containing sensory organ

Notch acts at two levels in the development of bristle-containing sensory organs. The first is in the selection of a single sensory precursor cell. Initially, all cells of the proneural clusters express both Notch and Delta but just one cell wins out. It possesses a more "effective" Delta that activates the Notch pathway of its neighbors and, through induction of E(spl), blocks expression of *chaete* and *scute,* thereby, shutting down their neurogenic program (Figure 19-12, panel (a)). Once the sensory organ progenitor cell has been established, Notch determines the subsequent cell lineage decisions so as to determine the destination of progeny, turning them into a bristle (shaft), socket-, glia-, neural-, or sheath cell (Figure 19-12, panel (b)). The first (asymmetric) division of the sensory precursor, leading to the progenitor cells, pIIa and pIIb, has been thoroughly investigated.

Scute: from *scutum*, the legion's shield, kneepan, scale, piece of bony armor in crocodile, sturgeon, turtle, armadillo, etc. Scute is a sex-linked gene which controls a stage in the development of a set of bristles, the most characteristic of which are the four on the scutellum (see Figure 19-1) which can be used as an index of activity of the scute locus. The number of bristles on the scutellum is what is meant by the "scute character." When scute mutates, the change in phenotype is clear-cut and large in extent. Normally there are four bristles on the scutellum; the number dropping to 0.5 (on average) in males and 1.5 in females in Sc1 mutants (Rendel, 1969).

Achaete: The bristles of the mechanoreceptors are named chaetes. In *Drosophila*, there are macrochaetes and microchaetes, present on the edges of the wings and on the dorsal site (notum) of its thorax. Loss of chaetes is referred to as "achaete."

In the prophase of cell division, just before the condensed chromosomes are aligned by the microtubule cytoskeleton, three proteins of the SOP accumulate on one side of the cell. These are; neuralized (E3-ubiquitin ligase), Numb (adaptor protein), and the AP-2 complex (marked by staining for α-adaptin) (domain architecture, Figure 19-10). Due to a defective Notch receptor, the daughter cell possessing the higher content of these three proteins loses its ability to receive signals, but has an abundant capacity to transmit, due to very effective trafficking of Delta (Le Borgne et al., 2005). As with Lap (epsin, see above) Numb is another almost linear (nonglobular) protein. It has an N-terminal PTB domain which, however, does not necessarily require a phosphotyrosine in order to bind to other proteins. Numb binds Notch and it binds Sanpodo. Its tail attaches to the ear segment of α-adaptin (AP-2 complex) (Figure 19-13). As a consequence, the daughter cell that expresses Numb looses cell surface expression of Sanpodo, which now concentrates in early endosomal vesicles due to selective uptake by Numb/AP-2 (Hutterer and Knoblich, 2005). After division, both Notch and Numb end up in late endosomes thus totally disabling a possible receptor function of the pIIb cell and sealing its lineage fate (Couturier et al., 2014). Moreover, Numb binds the RAM domain of Notch and this may prevent proteolytic processing or, if cleavage still occurs, it may even prevent binding of Nicd to its nuclear target Su(H) (equivalent of human RBPJ). On the contrary, the presence of neuralized accounts for very effective ubiquitylation and trafficking of Delta. As shown in Figure 19-8, this may lead to (a) focused cell surface expression of clusters of Delta, (b) association of essential cofactors, or (c) activation of Notch by pulling the inhibitory ankyrin repeats of Notch away from the S2-cleavage site (Le Borgne et al., 2005; Bray, 2006).

Note that in this example, Notch acts predominantly by inhibiting neural differentiation but this is not always the case. In mouse and embryonic stem cells, it acts to promote neural fates, a finding that underscores yet again the importance of cellular context in determining the outcome of signaling (Lowell et al., 2006).

NOTCH IN THE MAINTENANCE OF AN INTESTINAL STEM COMPARTMENT

Activation of Notch causes potent inhibition of differentiation in a number of developmental contexts. It has been associated with amplification of somatic stem cells, such as the neural and hematopoietic stem cells (Shen et al., 2004; Varnum-Finney et al., 2000). The intestine too has its stem cell compartments, present in the crypts (Figure 19-14). As described in Chapter 12, Wnt signaling plays an important role in driving proliferation of these stem cells (as well as their early progenitors). In the case of the small intestine, they do not differentiate until they are pushed out of the crypt and start climbing up the walls of the villi. They are shed on achieving the top. Here we indicate how Notch acts in concert with Wnt

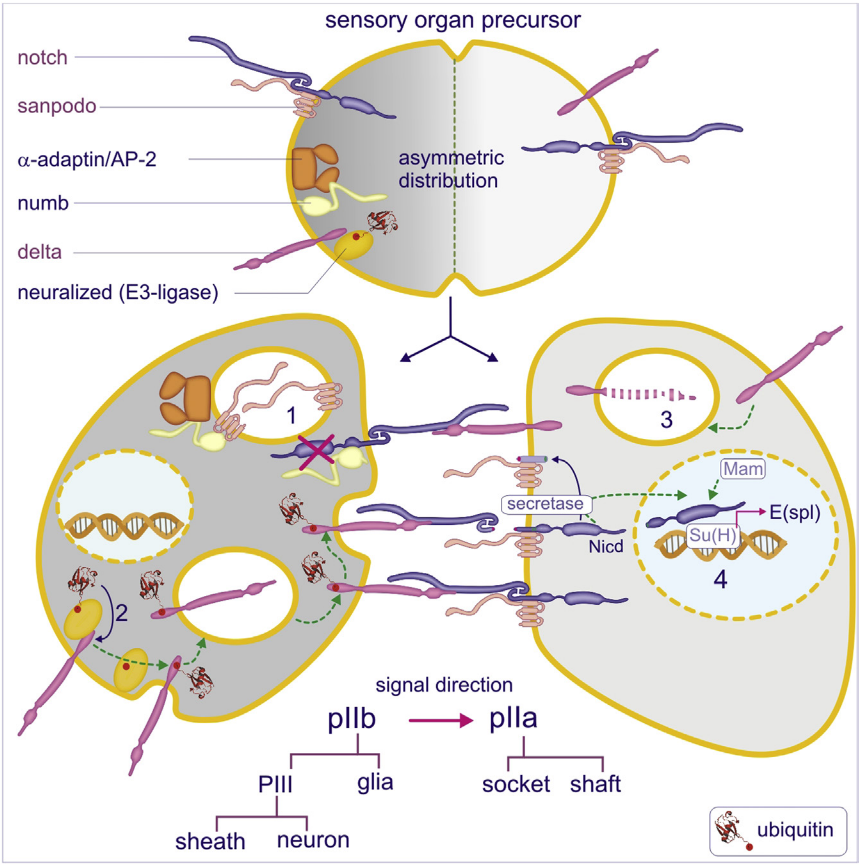

FIGURE 19-13 Asymmetric division of the sensory organ precursor cell determines cell lineage fate of its daughter cells. During the prophase of the cell cycle, the sensory precursor cells distribute AP-2, numb, and neuralized asymmetrically. The daughter cell having the highest concentration of these components efficiently expresses the ligand, Delta. Notch signaling fails in this cell due to removal of Sanpodo from the plasma membrane (1) which occurs through Numb/AP-2-mediated uptake of Sanpodo. Neuralized, on the other hand, stimulates the trafficking of Delta (2), which now activates the Notch exposed on the adjacent cell. This signal-receiving cell seems to have some difficulty in expressing its ligand (3). Notch-mediated gene expression (4) assures that the receiving cell, pIIa, takes on the lineage of socket and shaft while the descendents of the sending, pIIb, cells will provide the neuron as well as the glial- and sheath cell.

by preventing differentiation of both the stem cells and their early progenitors. Moreover, Notch signaling is vital to assure a balanced mixture of secretory and absorptive cells.

The role of Notch in the development of intestinal crypts is well indicated by the finding that the intestines of zebrafish that are mutant for DeltaD (ligand) have an increased number of secretory goblet cells, at the

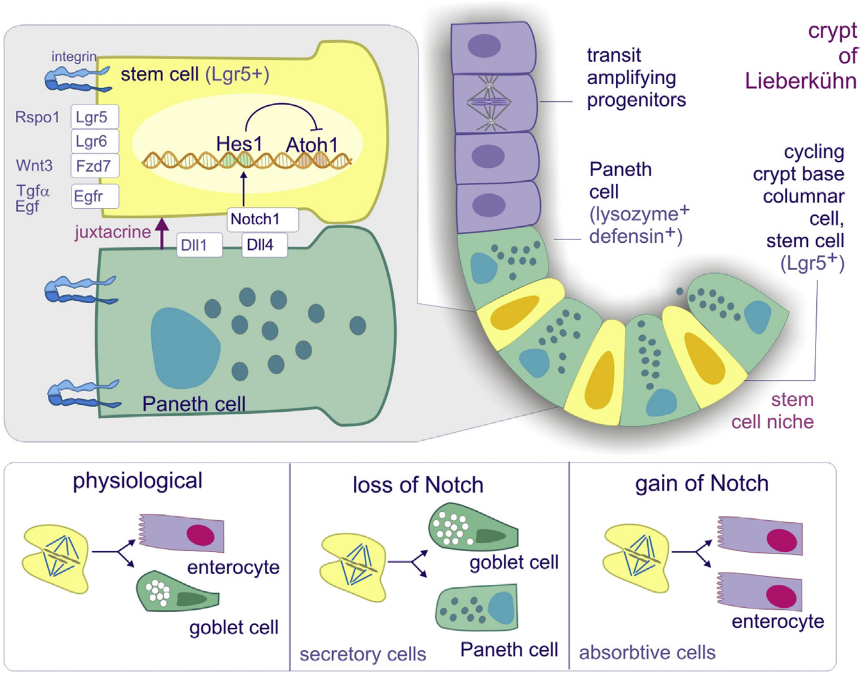

FIGURE 19-14 Notch regulates the size of the intestinal stem cell compartment and determines cell lineage of progenitor cells. The intestinal crypt, which harbors the stem cell compartment, is under the influence of EGF/TGFα, Wnt, and Notch. Notch prevents differentiation of the stem cell and its early progenitors. It also plays a role, possibly through lateral inhibition, in the lineage decision of the later-stage progenitor cells. Thus, "receiving" cells maintain an enterocyte (absorptive) phenotype, whereas the sending cell either turns into a goblet or Paneth cell (secretory cells). Loss of Notch therefore gives rise to a minute stem cell compartment and most of the progenitors turn into goblet or Paneth cells. Conversely, an excess of Notch prevents differentiation into secretory cells and leads to an excess of absorptive cells that continue to divide beyond the crypt region. In mice, Paneth cells express Delta4 (Dll4) which acts upon Notch1 of the neighboring "cycling crypt base columnar cell" (designated as the Lgr$^+$ intestinal stem cell). This brings about an enhanced expression of Hes1 which occupies the promoter region of Atoh1 and suppresses its expression. Atoh1 is an essential factor for the differentiation toward a secretory phenotype and its suppression contributes to the stemness of the cycling crypt base columnar cell. *Representation of the crypt of Lieberkühn adapted from Schuijers and Clevers (2012).*

expense of absorptive epithelial cells (enterocytes) (Crosnier et al., 2005). The default for a crypt progenitor cell is differentiation toward secretory goblet cells. To generate a balanced mixture of absorptive and secretory cells, lateral inhibition, mediated by Delta–Notch signaling, is needed. This is also true for mice in which multiple Notch pathway components are expressed. Mice lacking *Hes1* have few, if any, absorptive cells and reveal an excess of secretory and enteroendocrine cells. Induction of a constitutively active form of Notch1 in all the cells of the intestinal epithelium causes a loss of secretory goblet and a reduced number of enteroendocrine cells. Instead, there is an expansion of immature absorptive cells, some of which still divide far beyond their crypts (Fre et al., 2005). Adenomas, in which there is a highly active Wnt pathway, due to loss of function of the adenomatous polyposis coli (APC) protein, also employ the Notch pathway to suppress differentiation. They contain all necessary signaling components and express the Hes1 effector gene. Strikingly, when APC-deficient mice carrying numerous polyps were fed an inhibitor of γ-secretase, a large number of crypts regained a more or less normal histology, populated by numerous differentiated goblet cells (Milano et al., 2004; van Es et al., 2005). Lastly, transgenic expression of the Notch intracellular domain (Nicd) also results in a block of secretory cell differentiation and expansion of immature progenitors (Fre et al., 2005).

In search for stem cells of the intestinal tract it was shown that effective reconstitution of intestinal tissue, forming the so-called spheroids that contain both the cell types and the anatomical structures of crypts and villi, can be achieved through combining isolated crypt base columnar cells, recognized by expression of Lgr5, Olfm4, Tnfrsf19, and Cdca7 (van der Flier et al., 2009), with Paneth cells (recognized by expression of lysozyme and defensin A1) (Porter et al., 2002). It had already been determined that Lgr5$^+$ cells, earlier characterized as the cycling crypt base columnar cell, represent the intestinal stem cell, of which each crypt possesses roughly six copies and which divide every 24 h. However, this cell functions best in a stem cell niche where it is attached to a Paneth cell. In performing gene-expression profiles on stem- and Paneth cells it was noted that Paneth cells distinguish from the stem cell by elevated expression of Wnt3, Wnt11, EGF, TGFα, and the Notch ligand Dll4 (Sato et al., 2011). Wnt signaling instructs intestinal cells to adopt a proliferative progenitor phenotype (discussed in Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation, Section and "R-spondins potentiate the Wnt signal," Figure 12-18). In mice, Dll4 makes an important contribution by activating Notch1 and causing the suppression of expression, via Hes and Hey transcription factors, of epithelial cell fate determination genes, such as neurogenin (ngn), Achaete–Scute, and Atoh1 (formerly Math1) (van de Es et al., 2010). This contributes to the noncommitted progenitor state of the stem cell. Crypt base columnar cells attached to Paneth cells constitute the "stem cell niche." Production of Wnt is not the exclusive right of the Paneth cells, the ligands may derive from surrounding stromal tissue. Loss of Atoh1, under experimental conditions, eliminates Paneth cells and generates cells of the enterocyte lineage (Kim et al., 2012). Similarly, mice bearing a gain-of-function Kras allele retain their stem cells but lack Paneth cells and indeed one of the consequences of Kras is induction of expression of Hes1, a repressor that keeps Atoh1 down under (Feng et al., 2011). Once progenitor cells have detached from Paneth cells, they divide four or five times and then differentiate toward one of the four intestinal epithelial cell types, with a vast majority turning into enterocytes. In the ensuing differentiation program, expression of Atoh1 is essential for the transition toward secretory goblet cells (van de Es et al., 2010).

CROSS-TALK WITH OTHER SIGNAL TRANSDUCTION PATHWAYS

We end this chapter with examples that show that Notch interacts with other signal transduction pathways (or the other way round) and that this interaction occurs at different levels.

Cross-talk at the level of the membrane

Notch interacts with the Wnt pathway in *Drosophila* (Axelrod et al., 1996) and at least two pathway components are implicated. It binds Disheveled, an effector-protein immediate downstream of the Wnt receptor Frizzled (Fz) and it binds Armadillo (β-catenin), the component of the Wnt pathway that relays the signal into the nucleus (return to Chapter 12, "WNT Signaling and the Regulation of Cell Adhesion and Differentiation", in particular, Figure 12-3). Both interactions occur over a broad region of the intracellular domain of Notch. Gain-of-function mutants of Notch antagonize Wnt signaling. The observation that both Disheveled and Armadillo interact directly with Notch suggests that sequestration of essential signaling components forms the basis of this antagonism. Notch does not blunt the action of Wnt, but it may constitute an important buffer that raises the threshold level for Wnt signaling (Figure 19-15). In other words, Notch acts as a filter that cuts off low strength signals (noise) and this could perfectly serve to create sharp boundaries between responding and nonresponding cells in developing embryos (Brennan et al., 1999; Hayward et al., 2005).

Cross-talk at the level of gene expression

Notch cooperates with the TGFβ signal pathway at the level of induction of gene expression (Shen et al., 2004). In myoblast and adult neural stem cells, where addition of Notch and TGFβ prevent differentiation, Nicd and SMAD3/SMAD4 synergize by forming a protein complex that interacts with RBPJ. This leads to the higher levels of expression of Hes1 than either of the pathways alone can achieve. Hes1 protein, in turn,

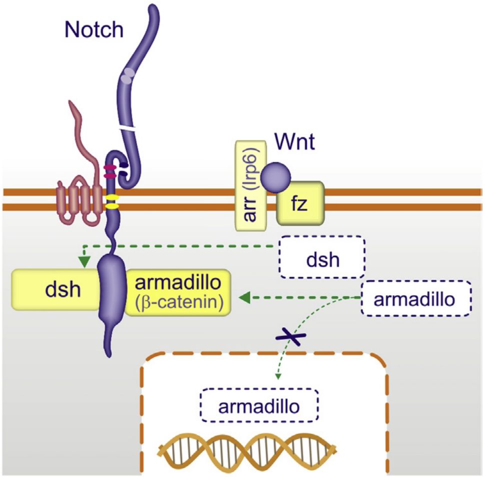

FIGURE 19-15 Cross-talk between Notch and the Wnt pathway. (a) In *Drosophila*, Notch binds Disheveled and Armadillo, components of the Wnt pathway, and thereby, increases the threshold of Wnt signaling. This may constitute a mechanism to filter out low strength signals (reduce noise).

prevents expression of myogenic factors such as the bHLH protein MyoD (Blokzijl et al., 2003).

A similar synergy at the level of transcription occurs between BMP4 and Notch but the context is different. Here we deal with vascular endothelial cells that show enhanced motility under the influence of BMP4 (member of the TGFβ family of cytokines). However, the motility of cultured cells ceases when the density rises to a level where frequent cell–cell contacts occur. During contact, Delta or Jagged-1 bind and activate Notch. The subsequently liberated Nicd associates with BMP4-activated SMAD1/SMAD4 complexes and with RBPJ. Together they induce high levels of expression of the bHLH protein HEY2 which, in turn, causes dedestruction of Id1 (Figure 19-16) (Itoh et al., 2004). Expression of Id1, also a bHLH, that sequesters transcription factors away from their favored promoter elements, is apparently essential for maintaining the motility of endothelial cells. It is known for its role in progression of the G1 phase of the cell cycle. In the context of migration, it suppresses expression of thrombospondin-1, a component of the extracellular matrix that acts to prevent new blood vessel formation (angiogenesis).

Cross-talk at the level of effector genes

In neural precursor cells, both the Stat and Notch pathways are implicated in the promotion of astrocyte differentiation. A surprising connection

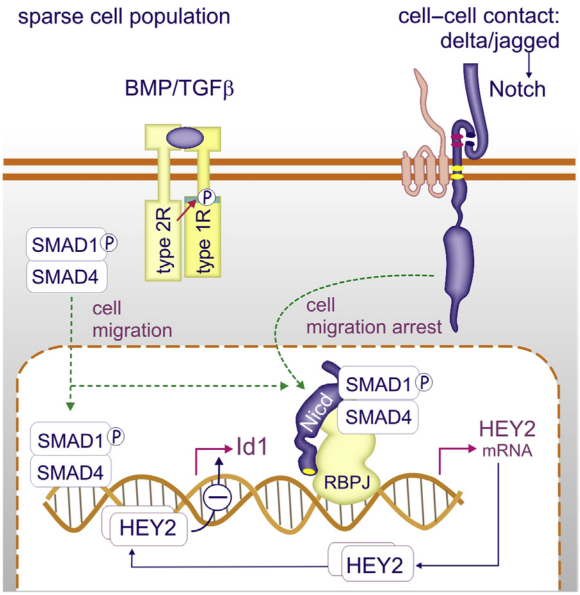

FIGURE 19-16 Cross-talk between Notch and the TGFβ signal pathway. The Nicd fragment of Notch interacts with phosphoSMAD1/SMAD4 and together they bind RBPJ and drive expression of Hey2. Hey2 dimerizes and binds the promoter of id1, leading to its suppression. With the onset of cell–cell contact, Delta-mediated Notch signaling arrests BMP4-mediated cell migration.

was found between the two pathways in which the transcription factor Hes1 has the role of gathering Stat3 with its kinase Jak2 (in a manner similar to the role of growth factor and cytokine receptors) (see Chapter 15, Figure 15-9). In short, Notch induces expression of Hes1 in cortical neuroepithelial cells of mouse embryos (Kamakura et al., 2004). Hes1 then binds either Stat3 or JaK2, most likely in the nucleus. Dimerized forms of Hes1 bring Stat3 together with Jak2. The ensuing phosphorylation causes Stat3 dimerization so that they bind DNA and thus act as transcriptional activators. This sequence of events shows that growth factors and Notch act in concert to induce Stat3-mediated cellular responses (Fiuza and Arias, 2007) (Figure 19-17).

Ras and Notch act in concert to determine cell fate in vulva development of C. *elegans*

As related in Chapter 10, the anchor cells of *C. elegans* present Lin-3 (neuregulin) which binds Let-23 (ERBB4) of the p6 cell and that this acts to determine the vulval cell phenotype. The vulval structure develops from

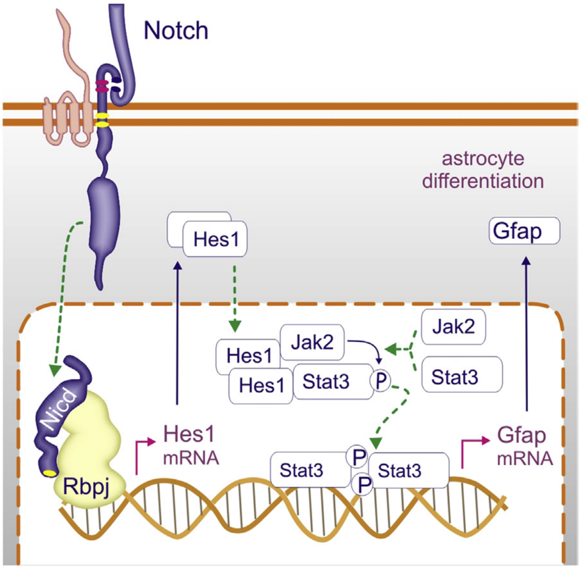

FIGURE 19-17 Cross-talk between Notch and the Jak/Stat signal pathway. Notch cooperates with Stat3 in the process of astrocyte differentiation. Nicd induces expression of Hes1 which facilitates Stat3 activation by acting as a platform that gathers both Stat3 and its kinase Jak2. The ensuing phosphorylation renders Stat3 transcriptionally active through dimerization. This sequence of events gives rise to expression of Gfap, an intermediate filament component and marker for astrocyte differentiation.

precursor cells that take on three different fates (Chapter 10, Figure 10-12). The first fate is reserved for p6 but since the anchor cells may also make weak contact with p5 and p7, or Lin-3 may be shed from the membrane and diffuse in the tissue, it is important that p6 can repress this route of development by its neighbors. This occurs through Let-60 (Ras)-mediated expression of Dsl-ligands which in turn activates the Lin-12 (Notch) pathway in both p5 and p7. The Notch signal blocks Let-23 (ERBB4) signaling in a number of ways so enabling the neighboring p5 and p7 cells to adopt the second fate. Let-60 (Ras) also prevents cell membrane expression of Lin-12 (Notch receptor) in p6 cells thus blocking a possible reciprocal action of p5 or p8.

Web resource

For more information about Notch and development of *Caenorhabditis elegans* go to URL: http://www.wormbook.org/.

NOTCH AND DISEASE

Defects in NOTCH2 or JAG1 (Jagged) are the cause of the Alagille syndrome type 2. This is an autosomal dominant multisystem disorder defined clinically by hepatic bile duct paucity leading to blockage of bile secretion (cholestasis, children having very pale feces) in association with cardiovascular (stenosis of lung arteries), skeletal (butterfly-shaped vertebral discs), and ophthalmologic manifestations. There are characteristic facial features (large forehead, deep embedded eyes, and protruding chin) and less-frequent involvement of the renal systems.

Reduced NOTCH1 and -3 expression is implicated in CADASIL syndrome, a subtype of inherited aortic disease, described as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Mutations in Delta-like 3 (DLL3) lead to spondylocostal dysostosis, with abnormalities including hemivertebrae and blocked vertebrae accompanied by deformities of the ribs which give rise to breathing problems and opportunistic respiratory infections (Fiuza and Arias, 2007).

Gain-of-function Notch mutations play a role in T-cell acute lymphoblastic leukemia. This form of leukemia is characterized by an excess of lymphoblasts (immature cells with swollen nucleus and little cytoplasm) which cause disease by crowding out normal cells in the bone marrow and by dissemination into other organs. It is most common in childhood with a peak incidence at 2–5 years of age, and another peak in old age. Acute refers to the relatively short time course in which severe pathological symptoms appear (weeks rather than months) (Ferrando, 2009). Mutations predominantly occur in the LNR-HD region and probably act by facilitating the cleavage of the S2-site. Another set of mutations concern the loss of the PEST motif which contains the phosphorylation sites necessary for recognition by the SCF E3-ubiquitin protein ligase. The Nicd fragment remains active for a much longer time span (Weng et al., 2004) (Figure 19-18).

Treatment of T-cell acute lymphoblastic leukemia with a γ-secretase inhibitor (MRK-003) brings about a reduction in expression of genes that are considered to be instrumental in the oncogenic transformation. With respect to cell metabolism, treatment causes a reduction in activity of PI 3-kinase and mTORC1, as well as a reduced expression of MYC. On the contrary, expression of PTEN, a phosphatidyl-3,4,5-phosphate phosphatase was increased. Together, these changes reduce the rate of protein synthesis. With respect to genes driving the cell cycle, less CCND3, CDK4, and CDK6 was observed, whereas expression of CDK inhibitors, such as CDKNB (p27kip) and cdkN1A (p21cip1), were found elevated, resulting in a cell cycle arrest.

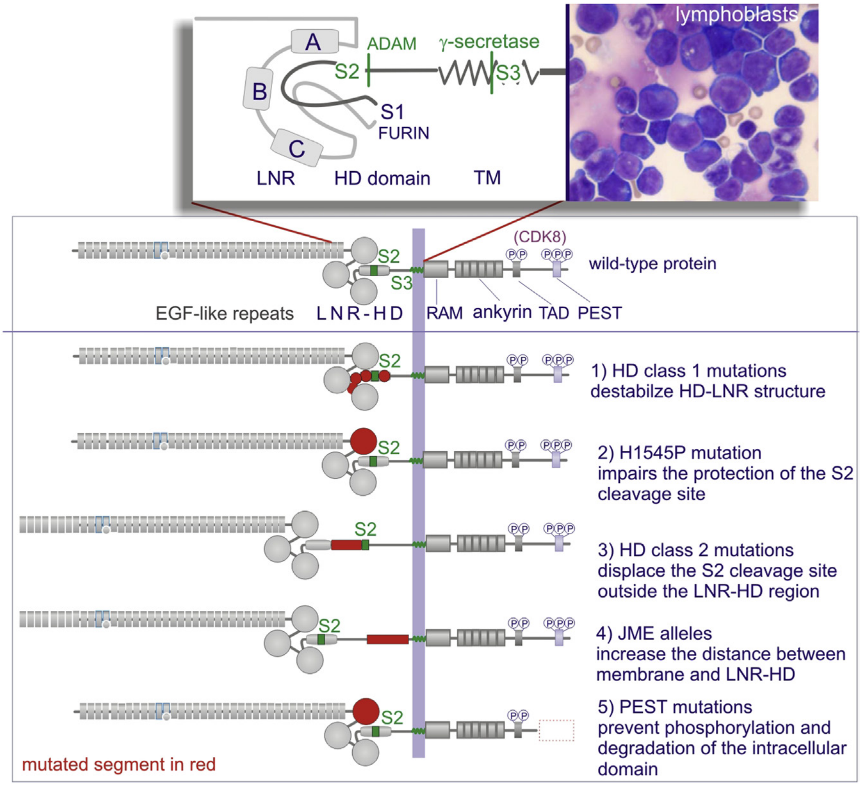
A
B
C
ADAM
γ-secretase
S2
S3
S1
FURIN
LNR
HD domain
TM
lymphoblasts
(CDK8)
wild-type protein
EGF-like repeats
LNR-HD
RAM
ankyrin
TAD
PEST
1) HD class 1 mutations destabilze HD-LNR structure
2) H1545P mutation impairs the protection of the S2 cleavage site
3) HD class 2 mutations displace the S2 cleavage site outside the LNR-HD region
4) JME alleles increase the distance between membrane and LNR-HD
5) PEST mutations prevent phosphorylation and degradation of the intracellular domain
mutated segment in red

FIGURE 19-18 Examples of mutations that enhance Notch signaling. These mutations occur in the LNR-HD segment and probably facilitate cleavage of the S2-site. Loss of the PEST motif prevents recognition by the SCF E3-ubiquitin ligase and thus destruction of the protein. The insert shows a bone marrow smear of T-cell acute lymphoblastic leukemia. *Image from German Cancer Research Center. Representation of Notch mutation sites adapted from Ferrando (2009).*

References

Arias, A.M., 2002. New alleles of Notch draw a blueprint for multifunctionality. Trends Genet. 18, 168–170.

Arnett, K.L., Hass, M., McArthur, D.G., Ilagan, M.X.G., Aster, J.C., Kopan, R., Blacklow, S.C., 2010. Structural and mechanistic insights into cooperative assembly of dimeric Notch transcription complexes. Nat. Struct. Mol. Biol. 17, 1312–1317.

Aguilar, R.C., Longhi, S.A., Shaw, J.D., Yeh, L.Y., Kim, S., Schon, A., Freire, E., Hsu, A., McCormick, W.K., Watson, H.A., Wendland, B., 2006. Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. Proc. Natl. Acad. Sci. U.S.A. 103, 4116–4121.

Axelrod, J.D., Matsuno, K., Artavanis-Tsakonas, S., Perrimon, N., 1996. Interaction between Wingless and Notch signaling pathways mediated by dishevelled. Science 271, 1826–1832.

Blokzijl, A., Dahlqvist, C., Reissman, E., Falk, A., Moliner, A., Lendahl, U., Ibáñez, C.F., 2003. Cross-talk between the Notch and TGF-b signalling pathways mediated by interaction of the Notch intracellular domain with Smad3. J. Cell Biol. 163, 723–728.

Bruckner, K., Perez, L., Clausen, H., Cohen, S., 2000. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature 406, 411–415.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J., Cerretti, D.P., 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385, 729–733.

Le Borgne, R., Remaud, S., Hamel, S., Schweisguth, F., 2005. Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in *Drosophila*. PLoS Biol. 3, e96.

Bray, S.J., 2006. Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7, 678–689.

Brennan, K., Klein, T., Wilder, E., Arias, A.M., 1999. Wingless modulates the effects of dominant negative notch molecules in the developing wing of *Drosophila*. Dev. Biol. 216, 210–229.

Le Borgne, R., Bardin, A., Schweisguth, F., 2005. The roles of receptor and ligand endocytosis in regulating Notch signaling. Development 132, 1751–1762.

Campus Ortega, J.A., Hartenstein, V., 1984. Early neurogenesis in wild-type *Drosophila melanogaster*. Roux's Arch. Dev. Biol. 193, 308–325.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., Rando, T.A., 2005. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433, 760–764.

Chen, M.S., Obar, R.A., Schroeder, C.C., Austin, T.W., Poodry, C.A., Wadsworth, S.C., Vallee, R.B., 1991. Multiple forms of dynamin are encoded by shibire, a *Drosophila* gene involved in endocytosis. Nature 351, 583–586.

Cubas, P., de Celis, J.F., Campuzano, S., Modolell, J., 1991. Proneural clusters of achaete-scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. Genes Dev. 5, 996–1008.

Couturier, L., Trylinski, M., Mazouni, K., Darnet, L., Schweisguth, F., 2014. A fluorescent tagging approach in *Drosophila* reveals late endosomal trafficking of Notch and Sanpodo. J. Cell Biol. 207, 351–363.

Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A., Lewis, J., 2005. Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. Development 132, 1093–1104.

Doe, C.Q., Goodman, C.S., 1985. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. Dev. Biol. 111, 206–219.

van de Es, J.H., de Geest, N., van de Born, M., Clevers, H., Hassan, B.A., 2010. Intestinal stem cells lacking the Math1 tumour suppressor are refractory to Notch inhibitors. Nat. Commun. 1, 18.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., Clevers, H., 2005. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435, 959–963.

Fryer, C.J., Lamar, E., Turbachova, I., Kintner, C., Jones, K.A., 2002. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev. 16, 1397–1411.

Fryer, C.J., White, J.B., Jones, K.A., 2004. Mastermind recruits CycC: CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol. Cell 16, 509–520.

Fischer, A., Gessler, M., 2007. Delta-Notch–and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res. 35, 4583–4596.

Ford, M.G., Mills, I.G., Peter, B.J., Vallis, Y., Praefcke, G.J., Evans, P.R., McMahon, H.T., 2002. Curvature of clathrin-coated pits driven by epsin. Nature 419, 361–366.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., Artavanis-Tsakonas, S., 2005. Notch signals control the fate of immature progenitor cells in the intestine. Nature 435, 964–968.

van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., van Es, J.H., Barker, N., Peters, P.J., van de Wetering, M., Clevers, H., 2009. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 136, 903–912.

Feng, Y., Bommer, G.T., Zhao, J., Green, M., Sands, E., Zhai, Y., Brown, K., Burberry, A., Cho, K.R., Fearon, E.R., 2011. Mutant KRAS promotes hyperplasia and alters differentiation in the colon epithelium but does not expand the presumptive stem cell pool. Gastroenterology 141, 1003–1013.

Fiuza, U.M., Arias, A.M., 2007. Cell and molecular biology of Notch. J. Endocrinol. 194, 459–474.

Ferrando, A.A., 2009. The role of NOTCH1 signaling in T-ALL. Hematol. Am. Soc. Hematol. Educ. Program 353–361.

Fortini, M.E., Artavanis-Tsakonas, S., 1994. The suppressor of hairless protein participates in notch receptor signaling. Cell 79, 273–282.

Gilbert, S.F., 1978. The embryological origins of the gene theory. J. Hist. Biol. 11, 307–351.

Gilbert, S.F., 1987. In friendly disagreement: Wilson, Morgan, and the embryological origins of the gene theory. Am. Zoologist 27, 797–806.

Ghysen, A., 1980. The projection of sensory neurons in the central nervous system of *Drosophila*: choice of the appropriate pathway. Dev. Biol. 78, 521–541.

Hartenstein, V., 2006. Genetic analysis of early neurogenesis: dedicated to the scientific contributions of Jose A. Campos-Ortega (1940–2004). Dev. Dyn. 235, 2003–2008.

Hambleton, S., Valeyev, N.V., Muranyi, A., Knott, V., Werner, J.M., McMichael, A.J., Handford, P.A., Downing, A.K., 2004. Structural and functional properties of the human notch-1 ligand binding region. Structure 12, 2173–2183.

Heitzler, P., Simpson, P., 1991. The choice of cell fate in the epidermis of *Drosophila*. Cell 64, 1083–1092.

Hawryluk, M.J., Keyel, P.A., Mishra, S.K., Watkins, S.C., Heuser, J.E., Traub, L.M., 2006. Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. Traffic 7, 262–281.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., Matsuno, K., 2004. *Drosophila* deltex mediates suppressor of hairless-independent and late-endosomal activation of Notch signaling. Development 131, 5527–5537.

Held, L.I., 2005. Imaginal Discs. Cambridge University Press, Cambridge.

Hutterer, A., Knoblich, J.A., 2005. Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in *Drosophila* external sensory organs. EMBO Rep. 6, 836–842.

Hayward, P., Brennan, K., Sanders, P., Balayo, T., DasGupta, R., Perrimon, N., Martinez, A.A., 2005. Notch modulates Wnt signalling by associating with Armadillo/beta-catenin and regulating its transcriptional activity. Development 132, 1819–1830.

Itoh, F., Itoh, S., Goumans, M.J., Valdimarsdottir, G., Iso, T., Dotto, G.P., Hamamori, Y., Kedes, L., Kato, M., ten Dijke, P.P., 2004. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. 23, 541–551.

Kidd, S., Lieber, T., 2002. Furin cleavage is not a requirement for *Drosophila* Notch function. Mech. Dev. 115, 41–51.

Kovall, R.A., Hendrickson, W.A., 2004. Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. EMBO J. 23, 3441–3451.

Kim, T.H., Escudero, S., Shivdasani, R.A., 2012. Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. Proc. Natl. Acad. Sci. U.S.A. 109, 3932–3937.

Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., Gotoh, Y., 2004. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat. Cell Biol. 6, 547–554.

Lissemore, J.L., Starmer, W.T., 1999. Phylogenetic analysis of vertebrate and invertebrate Delta/Serrate/LAG-2 (DSL) proteins. Mol. Phylogenet. Evol. 11, 308–319.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N.G., Israel, A., 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc. Natl. Acad. Sci. U.S.A. 95, 8108–8112.

Lecourtois, M., Schweisguth, F., 1995. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. Genes Dev. 9, 2598–2608.

Lai, E.C., 2002. Protein degradation: four E3s for the notch pathway. Curr. Biol. 12, R74–R78.

Lobley, A., Swindells, M.B., Orengo, C.A., Jones, D.T., 2007. Inferring function using patterns of native disorder in proteins. PLoS Comput. Biol. 3, e162.

Lowell, S., Benchoua, A., Heavey, B., Smith, A.G., 2006. Notch promotes neural lineage entry by pluripotent embryonic stem cells. PLoS Biol. 4, e121.

Morgan, T.H., 1919. The Physical Basis of Heredity. J.B. Lippincott Company, Philadelphia.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., Kopan, R., 2000. A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. Mol. Cell 5, 197–206.

Massari, M.E., Murre, C., 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell Biol. 20, 429–440.

Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M., Artavanis-Tsakonas, S., 1995. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development 121, 2633–2644.

Milano, J., McKay, J., Dagenais, C., Foster-Brown, L., Pognan, F., Gadient, R., Jacobs, R.T., Zacco, A., Greenberg, B., Ciaccio, P.J., 2004. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol. Sci. 82, 341–358.

Nichols, J.T., Miyamoto, A., Weinmaster, G., 2007. Notch signaling–constantly on the move. Traffic 8, 959–969.

O'Connor-Giles, K.M., Skeath, J.B., 2003. Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in *Drosophila*. Dev. Cell 5, 231–243.

Okajima, T., Irvine, K.D., 2002. Regulation of notch signaling by o-linked fucose. Cell 111, 893–904.

Okajima, T., Xu, A., Lei, L., Irvine, K.D., 2005. Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. Science 307, 1599–1603.

Okajima, T., Xu, A., Irvine, K.D., 2003. Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. J. Biol. Chem. 278, 42340–42345.

Olave, I., Reinberg, D., Vales, L.D., 1998. The mammalian transcriptional repressor RBP (CBF1) targets TFIID and TFIIA to prevent activated transcription. Genes Dev. 12, 1621–1637.

Owen, D.J., Collins, B.M., Evans, P.R., 2004. Adaptors for clathrin coats: structure and function. Annu. Rev. Cell Dev. Biol. 20, 153–191.

Poulson, D.F., 1937. Chromosomal deficiencies and the embryonic development of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 23, 133–137.

Poulson, D.F., 1950. Histogenesis, organogenesis and differentiation in the embryo of *Drosophila melanogaster* meigen. In: Demerec, M. (Ed.), Biology of *Drosophila*. Wiley, New York, pp. 168–274.

Poodry, C.A., Bryant, P.J., Schneiderman, H.A., 1971. The mechanism of pattern reconstruction by dissociated imaginal discs of *Drosophila melanogaster*. Dev. Biol. 26, 464–477.

Perry, W.L., Hustad, C.M., Swing, D.A., O'Sullivan, T.N., Jenkins, N.A., Copeland, N.G., 1998. The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. Nat. Genet. 18, 143–146.

Porter, E.M., Bevins, C.L., Ghosh, D., Ganz, T., 2002. The multifaceted Paneth cell. Cell Mol. Life Sci. 59, 156–170.

Radtke, F., Raj, K., 2003. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat. Rev. Cancer 3, 756–767.

Ramain, P., Khechumian, K., Seugnet, L., Arbogast, N., Ackermann, C., Heitzler, P., 2001. Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. Curr. Biol. 11, 1729–1738.

Rendel, J.M., 1969. A model relating gene replicas and gene repression to phenotypic expression and variability. Proc. Natl. Acad. Sci. U.S.A. 64, 578–583.

Simpson, P., 1994. The Notch Receptors. R G Landes & Co.

Schroeter, E.H., Kisslinger, J.A., Kopan, R., 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 393, 382–386.

Shen, J., Kelleher, R.J., 2007. The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. Proc. Natl. Acad. Sci. U.S.A. 104, 403–409.

Struhl, G., Adachi, A., 1998. Nuclear access and action of notch in vivo. Cell 93, 649–660.

Stern, C., Tokunaga, C., 1968. Autonomous pleiotropy in *Drosophilia*. Proc. Natl. Acad. Sci. U.S.A. 60, 1252–1259.

Shen, Q., Goderie, S.K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., Temple, S., 2004. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304, 1338–1340.

Schuijers, J., Clevers, H., 2012. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J. 31, 2685–2696.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., Clevers, H., 2011. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469, 415–418.

Tiyanont, K., Wales, T.E., Aste-Amezaga, M., Aster, J.C., Engen, J.R., Blacklow, S.C., 2011. Evidence for increased exposure of the Notch1 metalloprotease cleavage site upon conversion to an activated conformation. Structure 19, 546–554.

Taatjes, D.J., 2010. The human Mediator complex: a versatile, genome-wide regulator of transcription. Trends Biochem. Sci. 35, 315–322.

Tun, T., Hamaguchi, Y., Masunami, N., Furukawa, T., Honjo, T., Kawaichi, M., 1994. Recognition sequence of a highly conserved DNA-binding protein RBP-J kappa. Nucleic Acids Res. 22, 965–971.

Tutter, A.V., Fryer, C.J., Jones, K.A., 2001. Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. Genes Dev. 15, 3342–3354.

Usui-Ishihara, A., Simpson, P., 2005. Differences in sensory projections between macro- and microchaetes in Drosophilid flies. Dev. Biol. 277, 170–183.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W.S., Bernstein, I.D., 2000. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat. Med. 6, 1278–1281.

Weng, A.P., Ferrando, A.A., Lee, W., Morris, J.P., Silverman, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T., Aster, J.C., 2004. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306, 269–271.

Wolfe, M.S., 2006. The gamma-secretase complex: membrane-embedded proteolytic ensemble. Biochemistry 45, 7931–7939.

Zhou, S., Fujimuro, M., Hsieh, J.J., Chen, L., Miyamoto, A., Weinmaster, G., Hayward, S.D., 2000. SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. Mol. Cell Biol. 20, 2400–2410.

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